Characteristics of Genetic Factors that influence Gender in Atlantic salmon

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Abstract

Very little is currently known about how gender is established in Atlantic salmon, and environmental and commercial matters have increased interest in the life history strategies of this ancient fish. It is necessary to assess the sex ratios in populations of Atlantic salmon both before they head out to oceanic feeding grounds and when they return to spawn, before we can begin to understand the mechanisms that govern gender in this species. Much of the challenge faced by those studying gender in the Atlantic salmon lies in the problem of sexing juvenile salmon. It is currently not possible to sex Atlantic salmon without the need to sacrifice the individual. The principle aim of this project was to establish such a method. To this end, several approaches were taken to attempt to develop a non-destructive method of sexing juvenile salmon. The first comprises a PCR-based test to assess whether the *Sox9a* gene, known to be involved in vertebrate sex determination, is gender-specific in Atlantic salmon. The second strand of work involved the use of genetic markers identified as gender-specific in Pacific salmon to reveal whether any of these sequences segregated with gender in Atlantic salmon. In the final part of the study, a suppressive subtractive hybridisation technique was used to compare male and female Atlantic salmon cDNA, and isolate sequences unique to each. Results from the *Sox9a* study showed that the *Sox9a* gene is present in both males and females, and therefore not gender-specific in Atlantic salmon. One of the sequences identified from Pacific salmon showed female-specificity in one of the three strains of Atlantic salmon tested. The suppressive subtractive hybridisation technique successfully yielded two populations of differential sequences from male and female cDNA. The work done in this study thus partially achieved the aim of establishing a non-destructive gender test and lays the foundations of further work exploring gender in Atlantic salmon.

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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Abbreviations

AMH	anti-müllerian hormone
AMP	adenine monophosphate
ase	asense
ASL1	Atlantic salmon linkage group 1
ASW	avian sex-specific, W-linked
BKm	banded krait minor-satellite
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DAPI	4,6-diamidino-2-phenylindole
DMRT1	doublesex and mab-3 transcription factor 1
DNA	deoxyribonucleic acid
dpc	days post coitum
ECR	evolutionarily conserved region
EMT	epithelial to mesenchymal transition
ENS	enteric nervous system
ERα	oestrogen receptor alpha
ESD	environmental sex determination
ESS	evolutionarily stable strategy
FAO	Food and Agriculture Organisation of the United Nations
FET	female expressed transcript
FISH	fluorescence in situ hybridisation
FPT	female promoting temperature
GH-ΨY	growth hormone pseudogene Y
GSD	genetic sex determination
hCG	human chorionic gonatotropin
HIT	histidine triad motif
HINT	histidine triad nucleotide-binding protein
HMG	high mobility group
MHM	male mypermethylated region
mRNA	messenger RNA

miRNA	microRNA
MPT	male promoting temperature
mya	million years ago
NR	neural retina
PCP	pigment cell precursors
PCR	polymerase chain reaction
PCWH	peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, waardenburg syndrome, and hirschsprung disease
PNS	peripheral nervous system
RAPDs	randomly amplified polymorphic DNAs
RPE	retinal pigment epithelium
RNA	ribonucleic acid
RNAi	RNA interference
SEX	sex determining locus
SF1	steroidogenic factor 1
SOX	Sry-related HMG box
SRY	sex-determining region Y
SSH	suppressive subtractive hybridisation
TDF	testis determining factor
TESCO	testis-specific enhancer of sox9
TSD	temperature-dependent sex determination
UV	ultraviolet
WPKCI	W-linked protein kinase C inhibitor
ZFY	zinc-finger Y chromosomal protein
ZPKCI	Z-linked protein kinase C inhibitor

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1. Introduction

"X and Y chromosomes determine sex – the presence of a Y chromosome indicates a male and conversely, its absence a female"

This statement can be seen as a simple statement of fact when applied to human gender, but it can also be construed as a grossly naïve generalisation of the dynamics of gender differentiation when applied in the context of the wider animal kingdom. Certainly, when considering human, or indeed mammalian gender, there will probably not be a truer sentiment than the one aforementioned. Outside of this category however, this statement would stir up a cacophony of protests from geneticists around the world. Diversity in the dynamics of gender differentiation in the animal kingdom is so vast, that attempting to quantify all the similarities, differences, and nuances would be futile at best. However, comparing and quantifying the mechanisms in place for related species can be useful in understanding how and why something has evolved to be the way it is. However, furthering scientific knowledge may not be the only motivation for understanding gender differentiation. For example, in species with economic or commercial value such as fish, where males are generally more sought after as ornamental entities, and females are generally more sought after as food, a deeper knowledge of the mechanisms involved in sexual differentiation could help those in the fish farming, or *aquaculture*, industries to maximise the economic productivity of their businesses. One such species, where the study of its mechanisms of sexual differentiation would be both academically interesting and potentially commercially profitable, is the Atlantic Salmon *(Salmo salar)*.

This thesis is about genetic factors that are associated with sex in fish, and more specifically, in the Atlantic salmon, *Salmo salar*. In this general introduction we discuss why sex is worth studying and why fish in particular are a good model system in which to study it.

Arguably the most important and characteristic feature of living things is that they reproduce. In higher animals this virtually always involves sex, and so the scientific study of sex is fundamental to understanding the complexity of life. The irony is that it is far from clear why sex is necessary.

To see why this is so we need to define some terms. Sex in its broadest sense covers the process whereby two organisms come together to produce offspring. The actual process of mating requires that each participating organism contribute something to the incipient new organism. For the purposes of this discussion we will assume that the contribution is a cell or at least a nucleus of a cell, and in making this stipulation we rule out of consideration bacterial "sex" in which the contribution of one partner is only DNA.

As is well-known, the process of producing cells for use in mating is a complex one involving a special kind of nuclear division termed meiosis. The effect of meiosis is to produce a cell which contains half the diploid number of chromosomes (1 copy of each chromosome, neither more nor less), and the fusion of two such cells from different organisms produces a diploid zygote. Usually these "germ" cells are of different sizes (anisogamous), the smaller kind of cell is termed "sperm" or "spermatocyte" whereas the larger is termed "egg" or "oocyte". Dire consequences

ensue when defective germ cells fuse and there are many "choke-points" during development where the zygote may become unviable. Avoiding problems during the development of the zygote generally necessitates either the production of vast numbers of gametes (and hence zygotes), or some degree of parenting, an activity which uses a great deal of energy and involves major risks to the parenting organism(s).

Given such a dangerous and cumbersome process it seems obvious that if there were a safer and less cumbersome alternative it would be favoured by Natural Selection. This is the problem: there IS such an alternative, but it is NOT favoured by selection in most organisms! The alternative is asexual reproduction or parthenogenesis. In basic terms this involves females producing offspring without the need for a male contribution. The details of how this happens vary from species to species and are reviewed in Maynard Smith (1978). Parthenogenesis is observed in many groups of organisms, from Rotifers up through Insects and Fish, even as far as Birds.

Parthenogenetic lines are not long-lived in evolutionary terms, and this has led to two views of why sex is so prevalent. The first view is that group selection has resulted in the extinction of parthenogenetic lineages in the past. This would have to mean that truly-sexual reproduction has, or has had, some enduring advantage over asexual reproduction. The other view is that sexual reproduction is an evolutionarily stable strategy (ESS), which is a concept coined primarily by Maynard Smith (1973) to embody the notion that Darwinian Evolution involves competitive interactions similar to those seen in games. As Von Neumann showed, for many games (the classic examples being rock-paper-scissors and Prisoner's dilemma) it is possible to define a

winning strategy, deviation from which will often lead to defeat. Strategies of this kind are termed stable strategies.

Given the fact that there are both sexually and asexually-reproducing animals alive today it is not possible to distinguish between these hypotheses, but it is likely that Game Theory can contribute to our understanding of sex. An example of this is gender ratio, and a key concept here is *investment*. During sexual reproduction each partner contributes an investment of material to an offspring. This offspring represents the only future that either parent's genes have. Therefore, Natural Selection will strongly favour against heritable traits that act against the production of viable offspring. One such trait can be gender. If a lineage reproduces exclusively sexually, then organisms within it MUST find a partner. If there are two genders in the lineage, this means that the rarer gender is at an advantage. As a result, sex ratios are predicted to approximate to 1:1, since if they do not (for example, if there are more females than males), selection will favour organisms that produce males, right up to the point where there are more males than females, at which point selection will favour organisms that produce females.

However, exactly how this logic will play out in practice is inseparable from the actual mechanisms by which gender is determined. As is widely known, in humans and other mammals gender is genetically determined by the inheritance from the male parent of either his Y-chromosome (in which case one becomes male) or his X (in which case one becomes female). The female parent may donate either of her X-chromosomes but this contribution will not determine gender. Once gender is determined (at the moment of conception) it cannot be changed in any simple way.

Therefore, sex ratios are essentially fixed in organisms such as ourselves by genetic factors. The example of human trans-sexuals shows that people aren't always happy with the genders assigned to them by their genes, which may lead to some quite desperate attempts to escape at least the superficial constraints of genetic gender.

Nevertheless, as we shall see below, in other organisms decisions about gender are often taken by interactions between genes and the environment. This means that in principle they may be more easily reversible in other organisms than in ourselves. To the extent that this is so we might expect that much more subtle modulations of life history strategy may be observable in other organisms. In the most extreme case one might even observe an individual changing gender several times during its life-time, so as to maximise its reproductive success (this, and other cases are discussed in further detail later). Whether this is actually possible as opposed to theoretically desirable will, of course, depend on the practical details of how gender is determined, since not all biological processes are reversible.

Following on from the argument given above, a major motivation for the present study is to shed light upon gender determination as a life-history strategy, and apart from the academic, there are essentially three reasons why Atlantic salmon was chosen as the species in which to study this. The first reason is that while mechanisms of gender determination in Atlantic Salmon are poorly understood, they certainly appear to involve both biotic (likely but not certainly genetic/heritable) and environmental factors. The second is that fish as a group of organisms exhibit the widest variety of gender determination mechanisms known in any animal group (reviewed below). The

third is that Salmon gender determination is of practical importance both in ecology and aquaculture.

In the following sections of the introduction we review Salmon biology; give a taxonomy of gender determination mechanisms; and explain the ecological context of the study.

1.1 The Atlantic Salmon, *Salmo salar*

The majestic Atlantic Salmon, correctly apostrophised – some may argue – as the King of Fish, is an anadromous teleost species. This means that it spawns and spends its juvenile life in freshwater streams and rivers; gaining maturity and spending most of its adult life at sea; then returning to the river or stream where it was born only to spawn again. The word *salar* means 'the leaper', referring to the Atlantic salmon's magnificent characteristic ability to surmount waterfalls and other obstacles in its migration back to the stream of its origin. This name, and the first scientific description of Atlantic salmon were given in 1758 by Carolus Linnaeus, the great Swedish Taxonomist and botanist.

Fig. 1.1.1
The Atlantic Salmon, *Salmo salar*.

1.1.1 Life Cycle of Atlantic Salmon

The life cycle of the Atlantic salmon begins as thousands of eggs are laid by the hen (female) salmon in late autumn, in depressions in the riverbed gravel called ‘redds’. Development of the eggs begins right after they have been fertilised by the *milt* (sperm) of the cock (male) salmon, and in normal water temperatures will hatch after about 180 days. The fertilised eggs are normally pink or orange in colour, and will pass through the ‘eyed egg’ phase, in which the foetal fish’s eyes are clearly visible throughout its development through to its hatching.

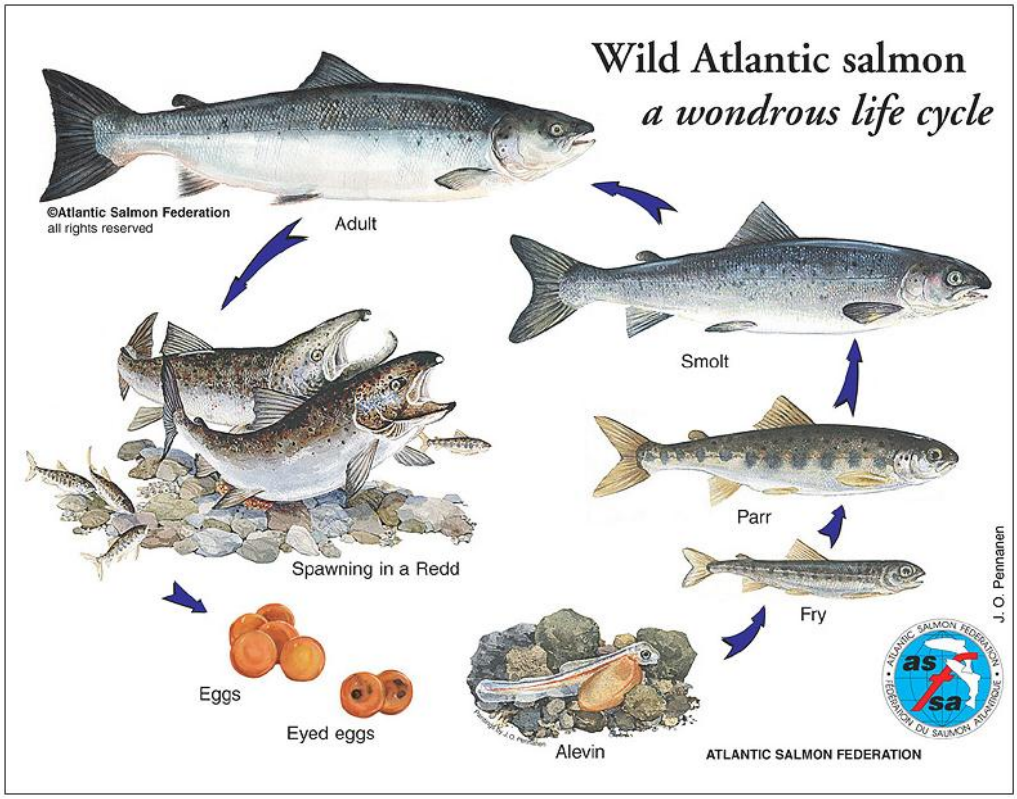


Fig. 1.1.2

The life cycle of the Atlantic Salmon.

From *the Atlantic Salmon Federation*, www.asf.ca.

The eggs hatch in the spring, and the <2cm long newly-borne fish are called *alevins.* At this stage, they have not yet completed their development, and look more like worms than fishes. They remain in the riverbed gravel for a few weeks for protection from predators, and feed on a yolk-sac that is attached to their bodies. When this yolk-sac runs out after 3-6 weeks, the alevins emerge from under the gravel as free-swimming fishes called *fry*. The fry immediately begin feeding on microscopic organisms, and quickly reach a length of 5-8cm at which point they develop into *parr*.

The young salmon now have distinct vertical markings, known as 'parr marks', along the length of its body. At one time, these markings were subject to debate among biologists debating whether the parr were, in fact, a different species and not young Atlantic salmon. The stripes act as camouflage so that the young salmon can blend in with the stream vegetation and remain unseen from predators. The parr remain in the stream for 1-4 years, depending on food availability and water conditions. After this time, they progress onto the next stage of their development, called *smolts*.

In springtime the more mature smolts, at a length of 12-24cm, undergo complex physiological and biochemical changes to enable them to live and feed in salt water conditions and the parr markings make way for a silvery sheen. It is at this time that they begin their migration downstream towards the sea. During this journey, certain characteristics of the stream are imprinted on the smolts' memory, allowing it to find its way back when it returns to spawn as an adult. The exact mechanisms of this remarkable phenomenon are still not fully known, and research is ongoing to help us to understand these mechanisms. The smolts spend several weeks, or even months in

the river estuaries before heading out into the ocean, where they will develop into adult salmon. Feeding on a rich seafood diet of crustaceans and small fish, they rapidly increase in size and weight, developing quickly into adulthood.

After just one year at sea, some of the more precocious salmon return to their rivers of birth in order to spawn. These not-quite-adult individuals are called *grilse* and average just 2-3kg on their return. The remaining adult salmon will spend a number of years at sea, reaching weights of up to around 50kg, before beginning the journey back to their rivers of birth for spawning in springtime. On reaching freshwater, adult salmon stop feeding, sustaining life by living off accumulated fat reserves. All of their energy is channelled into getting upstream to the locale in which they were born; and changing from their sleek silvery bodies into fish with humped bodies, losing their silver coats to a colourful breeding dress. The males also develop a hooked jaw called a 'kype', which is used in fighting in competition for females.

In late autumn – spawning season – the salmon prepare to spawn. The female salmon search for suitable nesting sites using a process called *exploring*, circling different stretches of the river, probing for unoccupied areas of appropriate water velocity, depth, and gravel type for their nests (Cavaller, 2004). When a suitable site has been identified, the female makes a depression in the gravel on the riverbed called a 'redd' by turning on its side and thrashing with its powerful body and caudal fin. The currents generated by this vigorous thrashing displace the gravel, creating depressions in the riverbed, usually between 10cm and 30cm in depth. While the females are selecting and building the sites for their nests, a hierarchy of suitors is established among the male population, with the dominant male occupying the position closest to

the female. From this position, the dominant male divides its time between courting the female and keeping the other males at bay through actual fighting, or by a series of threat displays to intimidate its rivals (Cavaller, 2004). This hierarchy is not stable, and the position of dominant male can be subject to change numerous times before spawning occurs.

Following a rather complex courting ritual involving, amongst others, 'false spawning' events (see Cavaller, 2004), the female (hen) finally spawns, and can lay as many as 1,500 eggs or more per kilogram of body weight into the redd. Simultaneously, the male releases its milt, often angling its body to direct the milt into the stream of eggs coming from the female. Finally the hen will replace the gravel to cover the eggs with additional thrusts of the tail. Sexually mature male parr and grilse normally manage to fertilise a percentage of the eggs – these precocious males are termed 'sneaker males', and are small and non-dominant. Not seen as a threat, and hidden in the gravel from the larger, dominant males, these vagabond interlopers emerge stealthily to release their milt, alongside that of the dominant male, into the nest of the spawning females.

Unlike Pacific salmon, where all the adults die after spawning, some adult Atlantic salmon do survive to return to the sea. These individuals are called *kelts*, and they eventually make their way back to the oceanic feeding grounds. Some make the journey back to the sea immediately, while others remain in freshwater pools for a few weeks to recover from spawning, and others still overwinter in freshwater habitats before heading out to sea. An action common to all survivors is that they will return

and respawn in the future. The fertilised eggs, however, stay in the nests until the following spring, when they will hatch and thus, the cycle begins again.

1.1.2 Distribution of Atlantic Salmon

Atlantic salmon are native to both sides of the North Atlantic Ocean, but limitations in the latitude range that populations inhabit are determined by water temperatures. The southern limit to this range is determined by the proximity of local water temperature to the lethal temperature for Atlantic salmon. This is, in the long-term 21-23°C, though they can withstand temperatures of up to 25°C for short periods. The northern limit is determined by the temperatures required for the development of eggs in time for the spring melt and where the feeding season before winter is long enough for fry to accumulate enough reserves to survive the following winter (Greenhalgh & Sutterby, 2005).

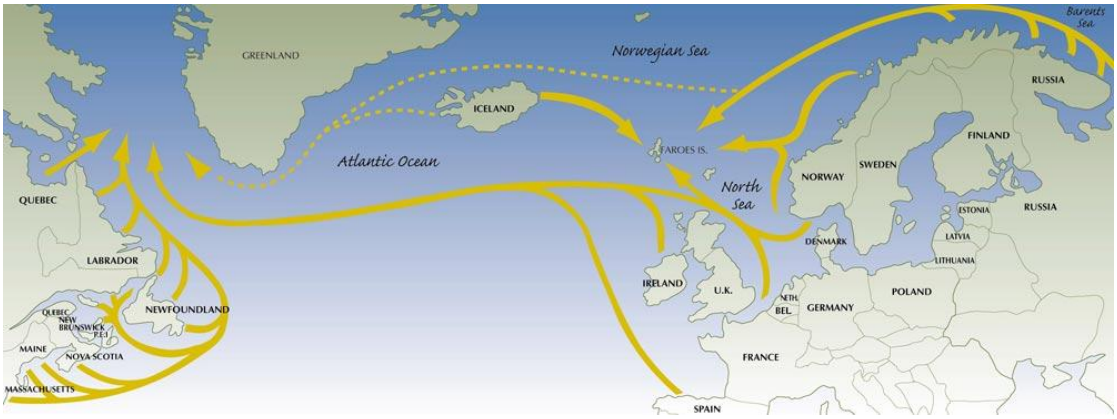


Fig. 1.1.3
Atlantic salmon migration routes to major feeding grounds
(Atlantic Salmon Federation, http://www.asf.ca)

On the eastern boundary of the range, populations of Atlantic salmon are found all along the European coast from the White Sea of Russia, down the coasts of Norway and Sweden, the Baltic Sea, including Finland and the former Soviet Union, continuing south around the British Isles and the coast of Western Europe to the border region of Spain and Portugal. This encompasses a range of 38°N to 71°N latitude on the Eastern side of the North Atlantic Ocean. Occurrence on the western side of the Atlantic basin incorporates the Ungava Bay in Quebec, Canada, the straits of Hudson and Davis and southern Greenland, then heading south in most rivers along the coasts of Labrador, Newfoundland, Quebec, and the maritime provinces to the Connecticut River of the United States. This represents a general range of 41°N to 60°N latitude, and as far north as 64°N (Kapisigdlit River), southern Greenland (Greenhalgh & Sutterby, 2005) on the Western side of the North Atlantic. Salmon from both sides of the Atlantic mainly converge in the waters off south-western Greenland or the Faeroe Islands; or in other minor oceanic and coastal feeding grounds where they spend most of their adult life, feeding on a rich marine diet of small fish and crustaceans such as plankton and krill (Atlantic Salmon Federation, 2010).

Although the regions discussed make up the native range of Atlantic salmon, there have been a number of attempts to establish Atlantic salmon in waterways elsewhere in the world. Most of these attempts have failed, and it is not clear whether populations of Atlantic salmon have successfully been established in the wild in these non-native waters. Consequently, most if not all Atlantic salmon caught outside of the

native regions can be assumed to be farmed escapees (see section 1.1.5), rather than wild individuals. For example, Atlantic salmon can be found in various river systems on the Pacific seaboard of the United States (McKinnell et al, 1997), and in Tasmania, Victoria and New South Wales in south-eastern Australia (Australian Government, Department of The Environment and Heritage), even though these regions are a long way from the Atlantic salmon's native range.

1.1.3 Evolutional History of Atlantic Salmon

"Nothing in biology makes sense, except in the light of Evolution"

– Theodosius Dobzhansky, 1973

Biologists interested in studying the evolutional history of river fish such as the Atlantic salmon have historically faced a rather large problem. There is simply not much of a fossil record. Where the remains of other species are buried in bottom sediments that eventually calcify the carcasses as fossils, those of river fishes are more likely to be either eaten by scavengers; physically destroyed by the action of running water; or are simply in unfavourable conditions for fossilisation to occur (Greenhalgh, 2005) – fossilisation being the product only of a number of factors coming together favourably. Comparing the structural and biochemical features of existing species, and cross-referencing knowledge of events over geological time has been the most plausible way of tracing the evolutionary line to *Salmo salar*.

At the root of the lineage, some 100 million years ago, a member of the **smelt** family (Family *Osmeridae*) experienced spontaneous chromosome doubling, and gave rise to the modern salmonid line. Smelts resemble small silvery salmonids, but lack the axillary process – a pointed flap above the base of the pelvic fin found in all fish further along the family tree. They also have an incomplete lateral line (which is complete in others).

The **whitefish** family (*Coregonidae*) is believed to have been the next to separate from the salmon's lineage, about 70 million years ago. Members of this family, like the smelts, have large scales but unlike the smelts, have minute teeth or are completely toothless. A key characteristic is that like the smelts, they scatter their eggs on the bottom of the lake or river. Further along the family tree, the eggs are covered in a gravel nest.

Next were the **grayling** line (*Thymallidae*), which branched away about 60 million years ago. Members of this family are the only ones in the salmon's family tree that cannot survive in sea water, suggesting that they evolved in a purely freshwater environment. Graylings also have large scales, but have minute teeth, a distinctly under-slung mouth and a very large dorsal fin where the fin base length is greater than the length of the head.

About 25 million years ago, a major branching from the main trunk of the tree occurred and gave rise to three genera: the **huchen** (*Hucho*); the **lenok** (*Brachymystax*); and the **charrs** (*Salvelinus*). The main line of the tree thereafter leads to all of today's trout and salmon.

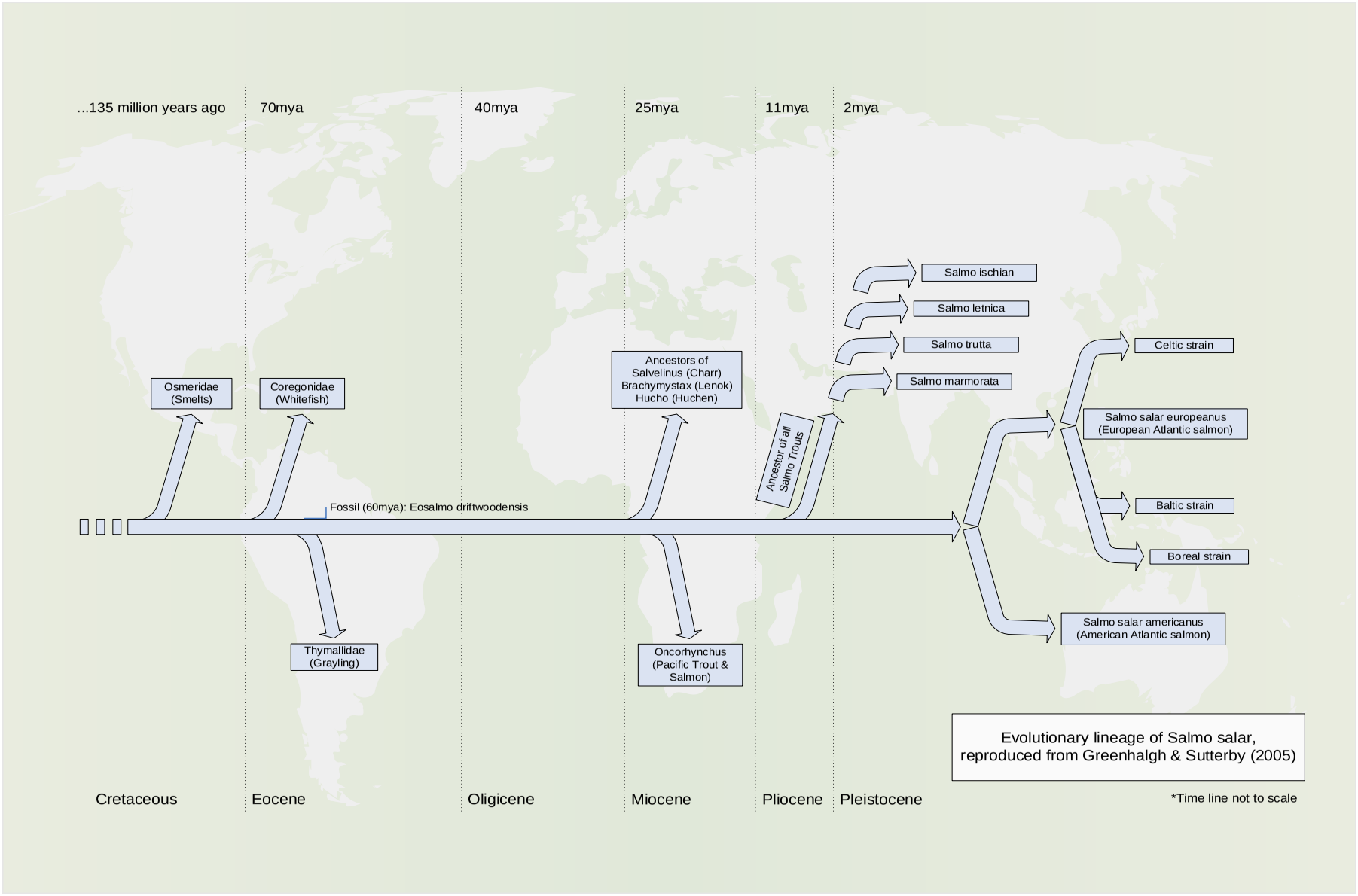
Another major branching occurred around 20 million years ago when the ancestors of the Atlantic basin trout and salmon became separated from their Pacific basin counterparts. This resulted in today's situation, where we have members of the genus *Oncorhynchus*, native in rivers and lakes draining into the Pacific Ocean, and members of the genus *Salmo*, native to rivers and lakes draining to the Atlantic Ocean and Mediterranean Sea.

Ancestors of the *Salmo* salmon and the *Salmo* trout parted around 10 million years ago. It is likely that the distinctly marked 'marbled trout', or *Salmo marmoratus* separated from the *Salmo* trout line around 2 million years ago, eventually producing the modern brown/sea trout, *Salmo trutta*. It is also possible that two other species of trout have since branched from the trout line in the past 100,000 years – *Salmo ischian* (only found in Lake Sevan in Armenia); and *Salmo letnica* (Lake Ohrid, Macedonia). However, most consider these to be merely subspecies of *Salmo trutta*.

It appears that the Atlantic salmon, *Salmo salar*, is at most some 10 million years old, but evolution does not stop, and has had implications since. It appears that around 600,000 years ago the American population of *Salmo salar* became isolated from the European population sufficiently enough to warrant subspecies status: *Salmo salar americanus* and *Salmo salar europaeus*. Even more recently, possibly in the last Ice Age of 70,000-10,000 years ago, the European subspecies became split into two distinct genetic strains – the Celtic Strain in south-western Europe (surviving the Ice Age in a glacial refuge in Iberia); and the Boreal Strain in the north-east (surviving in an ice-free lake in what is now the North Sea basin). Following the end of the Ice Age

the populations spread; the Celtic Strain northwards through France, the British Isles and southern Scandinavia; the Boreal Strain to Iceland, northern Scandinavia and Russia. The population of the Boreal Strain living in the Baltic Sea has become genetically distinct since the end of the last Ice Age, thought to be a consequence of Baltic salmon rarely venturing out to the Atlantic and interbreeding with Boreal Atlantic fish.

Fig. 1.1.4 (overleaf)
Evolutionary Lineage of *Salmo salar*


...135 million years ago
70mya
40mya
25mya
11mya
2mya
Osmeridae (Smelts)
Coregonidae (Whitefish)
Ancestors of Salvelinus (Charr) Brachymystax (Lenok) Hucho (Huchen)
Ancestor of all Salmo Trouts
Salmo ischian
Salmo letnica
Salmo trutta
Salmo marmorata
Celtic strain
Salmo salar europeanus (European Atlantic salmon)
Baltic strain
Boreal strain
Salmo salar americanus (American Atlantic salmon)
Fossil (60mya): Eosalmo driftwoodensis
Thymallidae (Grayling)
Oncorhynchus (Pacific Trout & Salmon)
Evolutionary lineage of Salmo salar, reproduced from Greenhalgh & Sutterby (2005)
Cretaceous
Eocene
Oligicene
Miocene
Pliocene
Pleistocene
*Time line not to scale

1.1.4 Related Species

Atlantic salmon (*Salmo salar*) is of the genus ***Salmo***, consisting of six species:

- *Salmo salar*
- *Salmo trutta*
- *Salmo platycephalus*
- *Salmo penshinensis*
- *Salmo letnica*
- *Salmo ischchan*

The genus *Salmo* is one of nine genera belonging to the family ***Salmonidae****:*

- *Salmo*
- *Salvelinus*
- *Oncorhynchus*
- *Thymallus*
- *Brachymystax*
- *Stenodus*
- *Prosopium*
- *Hucho*
- *Coregonus*

The family *Salmonidae* is of the order ***Salmoniformes***. The closest relatives to the Atlantic salmon are the *Salmo* species, and interesting comparisons can be made with members of the *Oncorhynchus* and *Salvelinus* genera.

Family	Subfamily	Genus	Species
Salmonidae	**Coregoninae**	*Stenodus*	(Sheefish and Inconnu)
		Coregonus	(Whitefish and Cisco)
		Prosopium	(Mountain whitefish)
	Thymallinae	*Thymallus*	(Grayling)
	Salmoninae	*Brachymystax*	*lenok* (Siberian lenok)
		Hucho	*hucho* (Huchen / Taimen)
			perryi
		Salmo	*salar* (Atlantic salmon)
			trutta (Brown trout / Sea trout)
		Salvelinus	*confluentus* (Bull trout)
			alpinus (Arctic charr)
			malma (Dolly varden)
			leucomaenis (White-spotted charr)
			namaycush (Lake trout)
			fontinalis (Brook trout)
		Oncorhynchus	*chrysogaster* (Mexican golden trout)
			gilae (Gila / Apache trout)
			clarki (Cutthroat trout)
			mykiss (Rainbow trout / Steelhead)
			masou (Masu salmon / Yamame)
			kisutch (Coho salmon)
			tshawytscha (Chinook salmon)
			nerka (Sockeye salmon / Kokanee)
			keta (Chum salmon)
			gorbuscha (Pink salmon)

Table 1.1

The *Salmonidae* Family, adapted from Watson, 1999.

1.1.5 Atlantic Salmon in Aquaculture

Aquaculture is the commercial production of aquatic species as food products. Over the years, aquaculture as a whole has become a very lucrative market, worth many billions of dollars (US) worldwide. In fact, it was estimated to be worth US$ 106 billion in 2008 (FAO, 2010).

The farming of Atlantic salmon under carefully controlled conditions has been prolific in both native and non-native waters. The main producer countries in the world today include Norway, Canada and the USA in the Northern hemisphere; and Australia and Chile in the Southern hemisphere (Fig. 1.1.5).

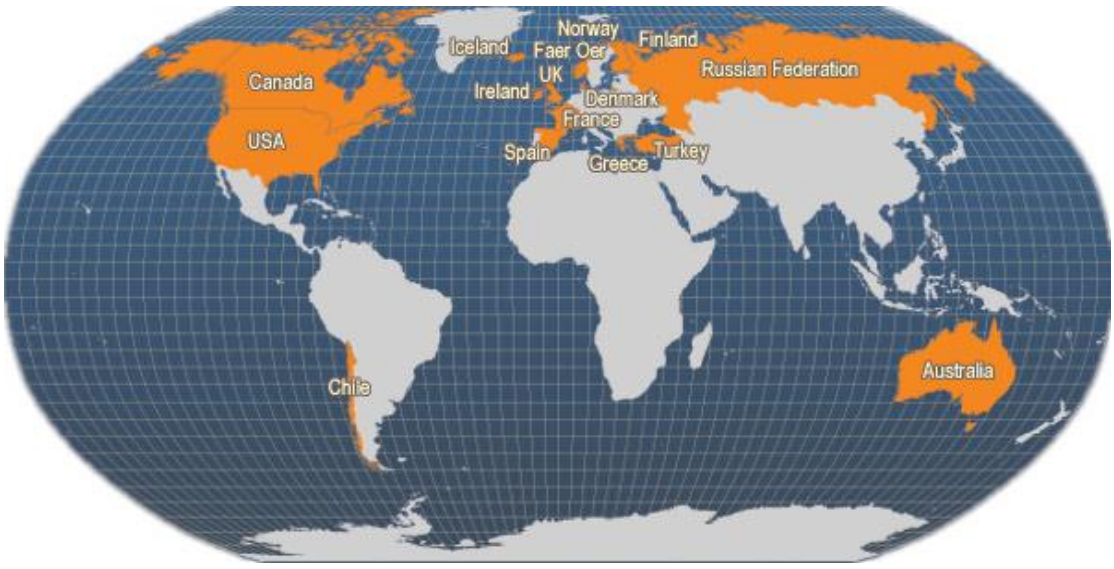


Fig 1.1.5
Main Producer countries of Atlantic Salmon
(FAO Cultured Aquatic Species Information Program, 2004-2010)

Figures from the FAO (Food and Agriculture Organisation of the United Nations) (2010) show that in 1988, a total of 110 599 tonnes of Atlantic salmon were produced, and was valued at US$ 668 million – 3.2% of the overall global aquaculture market. This was overshadowed by the figures for Rainbow trout, another salmonid species on the market, which showed production levels twice that of Atlantic salmon – 247 756 tonnes, at a value of over US$ 874 million and constituting 4.2% of the global aquaculture market. Atlantic salmon production was 25.3% of the total Salmonid production figures, and Rainbow trout 56.7%. The production figures for the two species represented 82% of all Salmonids produced by aquaculture, and 76% of the value of the Salmonid aquaculture industry.

Ten years later in 1998, a total of 668 227 tonnes of Atlantic salmon were produced worldwide, at a value of US$ 2.14 billion. In comparison, 437 816 tonnes of Rainbow trout were produced, at a value of US$ 1.27 billion. The Atlantic salmon industry's share of the global aquaculture market had increased to 5.7% of the total value, and the Rainbow trout's share had decreased to 3.0%. In terms of total production, Atlantic salmon accounted for 53.0% of Salmonids (51.7% in value), and Rainbow trout 34.0% (30.7% in value). Combined they accounted for 87.0% of total Salmonid production, and 82% of the Salmonid aquaculture industry's value.

By 2008 (latest figures currently available), Atlantic salmon production levels had reached 1 456 721 tonnes worldwide, and was valued at US$ 7.20 billion. By comparison, Rainbow trout production was at 576 289 tonnes, with a value of US$ 2.39 billion. This represents 7.3%, and 2.4% of the global aquaculture market respectively. Atlantic salmon accounts for 63.5% of total Salmonid production, and

67.5% of the Salmonid industry's value. Meanwhile, Rainbow trout accounts for 25.1% of total Salmonid production, and 22.4% of the industry's value. Combined, Atlantic salmon and Rainbow trout now represent 88.6% of total worldwide Salmonid production, and 89.9% of the Salmonid aquaculture industry's value.

But what does this mean? The headline statistics are as follows – in the 20-year period from 1988 to 2008:

- Atlantic salmon production has increased more than ten-fold (110 599 tonnes to 1 456 721 tonnes).
- The value of the Atlantic salmon aquaculture industry has increased more than ten-fold (US$ 668 million to US$ 7.20 billion).
- Atlantic salmon's share of the global aquaculture industry has increased from 3.2% to 7.3%.
- Conversely, Rainbow trout production has seen only a two-fold increase; its value has not quite made a three-fold increase; and its share of the global aquaculture industry has fallen from 4.2% to 2.4%.

These figures strongly suggest a rapid increase in the popularity of Atlantic salmon as a food product worldwide. With increasing global population, and increasing accessibility to foodstuffs previously considered difficult to acquire (due to advances in inter-continental transport and improving food preservation methods), the Atlantic salmon would appear to have firmly established itself as an important commodity on a global scale. This may have affected the popularity and therefore the value of the Rainbow trout industry, which has seen its share of the global aquaculture market almost halved.

Top ten Global Aquaculture Products by Value (US$ '000) 1988		
	Product	**Value (US$)**
1	Common Carp	1 778 840
2	Silver Carp	1 643 956
3	Fleshy Prawn	1 496 645
4	Giant Tiger Prawn	1 325 710
5	Japanese Eel	993 969
6	Rainbow Trout	874 696
7	Grass Carp	779 164
8	Bighead Carp	755 139
9	Pacific Cupped Oyster	694 626
10	Atlantic Salmon	668 312

Top Ten Global Aquaculture Products by Value (US$ '000) 1998		
	Product	**Value (US$)**
1	Giant Tiger Prawn	3 215 348
2	Silver Carp	2 633 711
3	Grass Carp	2 459 942
4	Common Carp	2 246 243
5	Atlantic Salmon	2 142 602
6	Japanese Carpet Shell	1 797 787
7	Roho Labeo	1 411 782
8	Bighead Carp	1 296 114
9	Rainbow Trout	1 274 529
10	Whiteleg Shrimp	1 019 747

Top Ten Global Aquaculture Products by Value (US$ '000) 2008		
	Product	**Value (US$)**
1	Whiteleg Shrimp	8 985 289
2	Atlantic Salmon	7 204 152
3	Grass Carp	4 797 279
4	Silver Carp	4 786 195
5	Common carp	3 696 415
6	Chinese Mitten Crab	3 608 126
7	Giant Tiger Prawn	3 349 552
8	Catla	3 303 124
9	Nile Tilapia	3 208 561
10	Japanese Carpet Shell	3 185 467

Table 1.2

Top Ten Global Aquaculture Products by Value (US$ ‘000) in 1988, 1998 and 2008.

(Data sourced from FAO Fisheries Statistics, 2010)

Farming of Atlantic salmon in the United Kingdom began in the 19th century as a means of stocking waters with parr in order to enhance wild returns for anglers (FAO Cultured Aquatic Species Information Programme, 2010). In the late 1960s, experimental work by *Unilever* and others in Norway led to the beginnings of the commercial production of Atlantic salmon in the 1970s. Over the next two decades the United Kingdom's Atlantic salmon aquaculture industry would grow very rapidly, especially in Scotland (Fig. 1.1.6), which is responsible for 80% of the total aquaculture output from the UK. Latest figures show that 128 606 tonnes of Atlantic salmon were produced by Scottish fish farms in 2008. This marks the continuation of a trend whereby production levels have remained at around 130 000 tonnes annually since 2005, with Marine Scotland Science – formerly Fisheries Research Services – (2009) suggesting that the industry is consolidating and stabilising at manageable production levels.

Despite the failure of attempts to establish wild populations of Atlantic salmon in Australia (Love and Langenkamp, 2003), the aquaculture industry here has prospered over the years. Eggs from Nova Scotia, Canada were first introduced in Tasmania at around 1984. With favourable sea temperatures and relative isolation, the area avoids some of the major infectious disease complications, and the industry has grown rapidly (FAO Cultured Aquatic Species Information Programme, 2010). In its first year of Atlantic salmon production, South Australian fisheries reported figures of 64 tonnes, valued at AU$600,000 in 2001-2002; and Tasmanian fisheries reported figures of over 14,000 tonnes for salmonid production – consisting of Atlantic salmon and ocean trout – valued at AU$111 million in the same period. This was up from 11,000

tonnes and 13,000 tonnes in the previous two years respectively (Australian Fisheries Statistics, 2002).

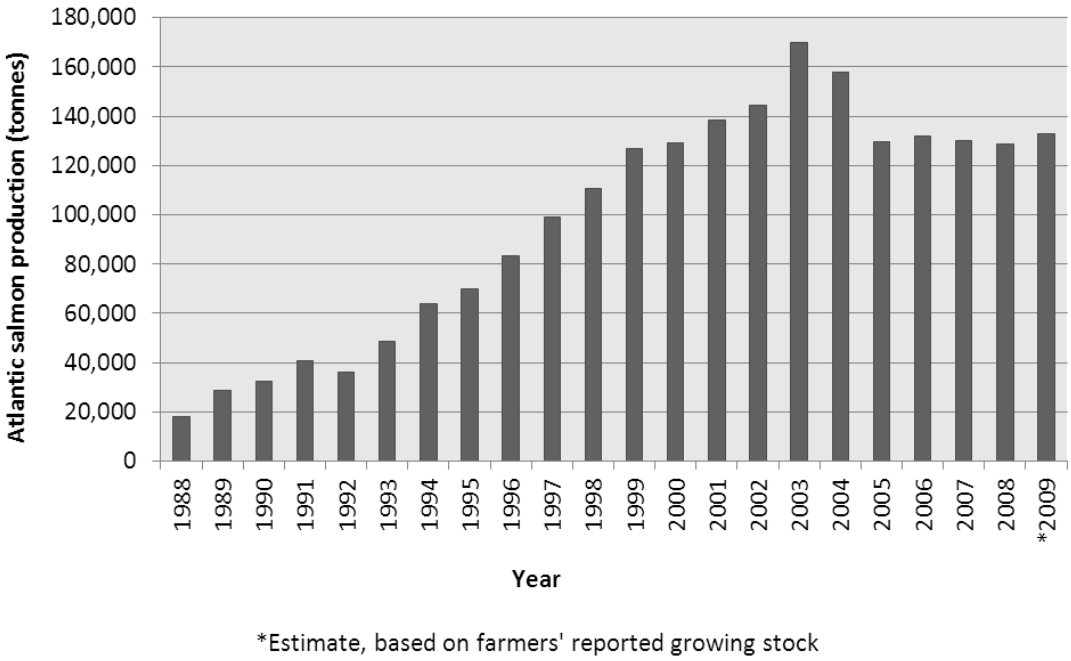


Fig 1.1.6
Atlantic salmon production in tonnes during the period 1988-2009.
(Data from Marine Scotland Science, 2009)

Elsewhere, the most rapidly growing supplier of farmed Atlantic salmon is Chile. Since the introduction of eggs from Norway and Scotland in the early 1980s, the Chilean Atlantic salmon industry has benefited from the country's low production and labour costs, thus accommodating effective competitiveness with the more traditional Atlantic salmon producing countries in the Northern hemisphere (FAO Cultured Aquatic Species Information Programme, 2010).

As an industry worth more than US$ 7.2 billion worldwide (and there is no reason to believe that it has not continued to grow in the last three years), it is clear that anything with a significant effect on the production of Atlantic salmon will have far-reaching consequences, be they economic (in revenue for the economies of producer countries); social (in jobs for workers in the industry); or environmental (in the possible effects of aquaculture on the natural world). For this reason, it is important that all operational decisions made within the industry are carefully considered. It is also for this reason that we must realise the importance of learning as much about this valuable organism as we can.

The farming of Atlantic salmon has historically been, and still appears to be, a rapidly-growing, and highly lucrative industry. Despite the difficulties encountered in establishing 'new' wild populations of Atlantic salmon outside of its native range, aquaculture of this highly popular food-fish has evidently flourished in locations all over the world, where favourable conditions prevail. Due to the popularity of this fish, those who are involved in breeding Atlantic salmon commercially are understandably very interested in ways of improving the commercial viability of their stocks. Having the ability to select for the more favourable traits in the salmon at a young age is undoubtedly an advantage, as this means they can concentrate their efforts on the more desirable individuals. To this end, work has commenced to try to understand the genetics and characteristics of the Atlantic salmon at a deeper level, in the first steps towards gaining this ability to select for commercially favourable traits.

1.1.6 Salmon, Humans, and Science

Historically a source of fascination to human beings, there are a variety of reasons why salmon are held in such high regard by people from all walks of life. Likely to be of primary importance is the fact that they are a large, flavoursome fish that is highly abundant where conditions are favourable. An important source of food for those that lived in its native range, and later sold as a luxurious commodity to those outside of it, one does not have to stretch the imagination to appreciate the status bestowed upon the Atlantic salmon as the 'King of fish', in times where modern technology had not yet made the provision of non-native foods a simple process. More recently, the curious and dramatic events of the Atlantic salmon's life cycle have come to light – a process driven in part by science and its quest for knowledge; and in part by its importance as a game fish, where knowing the best times of the year to land the best fish is important to recreational fishermen everywhere. More recently still, declining salmon populations have become an icon for post-modern discontent with the relationship between humanity and nature.

Something that is remarkable, given the long relationship between humans and salmon, is that little is known about key aspects of their biology. Among these areas of ignorance is the question of how gender is established in this fascinating fish. In recent times, this question has become increasingly important. Not only would it be scientifically interesting to understand the intricacies of Atlantic salmon gender, but two other reasons have also come to the fore: environmental; and as has already been mentioned, commercial.

The environmental concern has arisen because some long-term studies have indicated a certain degree of feminisation in Atlantic salmon populations. Generally, this can be seen as one of two things: either proportionally fewer males are seen than females, or the animals in general present more female characteristics. This concept of 'feminisation' has been offered in the popular press as evidence for the baleful effects of humankind upon the natural world. Clearly, in order for us to begin to understand this intriguing phenomenon, it is required that we understand how salmon gender is determined naturally.

The commercial concern is a somewhat simpler affair. Female fish are generally larger than male fish of the same age, so aquaculturists would ideally like to raise stocks that are predominantly or exclusively female for the market. There are currently very effective methods for feminising salmonids through the judicious application of hormones (discussed later) (Piferrer and Donaldson, 1992). However, in today's consumer climate, there is increasingly strong market resistance to hormone-treated food, and furthermore, it is not possible to claim the coveted 'organic' label for fish that have been subject to endocrine treatment such as the one mentioned. If one could determine which individuals would mature to be males and which to be females at an early stage in development, this difficulty could be overcome.

Both of these issues are further confounded by the difficulty of sexing juvenile salmon. Essentially, it is impossible to identify the gender of a live juvenile salmon (parr/smolt stages) by external examination alone. Even in adults, positive identification is not always assured. This is discussed in more detail later.

In terms of science, gender and certainly the mechanism by which it is determined has long been a subject of interest to humans. Going back to classical times, the poet Lucretius (in De Rerum Natura) postulated that originally, humans were eight-limbed creatures with three genders (male, female, and hermaphrodite – the latter being the most common), but that the angry Gods had split them into their present four-limbed state. From that time forward, the fable continued, men and women have been seeking physical reunion with their 'other half', usually but not always members of the opposite gender. Even earlier, Aristotle believed that gender was determined at conception – by the conditions under which intercourse was performed. Of course this view is not now held, but the scientific ideas underlying it have led to the modern picture of gender determination. Namely, that it is a genetic phenomenon involving the inheritance of Mendelian characteristics that encode a cascade of events leading to either male or female development. In the most widely-studied systems (humans and other mammals) the inheritance of a Y chromosome from the father leads to male development. Conversely, inheritance of the father's X chromosome leads to female development. Inheritance from the maternal element does not bear any significance for gender, as the mother always donates an X chromosome by default. When mutations in the genes encoding the gender-determining cascade occur, this picture suddenly becomes less clear, and gender can really become indeterminate. It is through analysis of these 'sex-reversed' individuals that the present understanding of gender determination has been elucidated.

This mechanism of gender determination is not uniform throughout the animal kingdom. In fact, outside of the mammalian system, the mechanisms at work are generally very poorly understood. What is evident is that in many of the other animal

groups, gender is flexible, with controlling factors being environmental, hormonal, or even social. In the highly competitive nature of the mating season in all animal species, it is not surprising that the vast majority of successful 'mates' are what one may deem 'the most fertile', 'the strongest', 'the most colourful', or even 'the least ridden with disease'. Whatever the criteria, individuals that display or possess the most desirable traits are most likely to successfully find a mate, and therefore produce offspring. Those that remain are inexorably at risk of being left by the wayside – unable to continue their lineage, to distribute their genes onto the next generation. A true reflection of Darwin's theory of Natural Selection, one cannot dispute. For these individuals, from a life-history point of view, there may be an advantage to be had in maximizing their potential for procreating by 'working the system' – perhaps in becoming the rarer or more sought after gender, the chances of finding a mate are more assured. This kind of sexual plasticity is particularly manifest in fish, one of which, the Atlantic salmon, *Salmo salar*, forms the main focus of this thesis.

We have already briefly visited the mating habits of the Atlantic salmon (the interested reader is directed to an excellent thesis on this topic by Cavaller, 2004), and mentioned the occurrence of 'sneaker males'. It would seem likely that there is a definite life history pay-off attached to sexual plasticity for Atlantic salmon – for those individuals that cannot compete with the larger, more aggressive males for mates, would it not make sense to develop alternative, more innovative ways to ensure the continuity of one's genetic lineage? Be it by becoming sexually mature at a much younger age – as a precocious parr for example, thus being able to 'sneakily' fertilize a proportion of eggs without being deemed a threat by the larger males; or simply by becoming female and being fought over, rather than have to fight, for the right to

mate. Either method would arguably enhance an individual's chances of producing offspring. It is well documented that sexually mature, precocious parr are successful in fertilizing a percentage of eggs, but what about the idea of an individual changing gender to increase its chances of finding a mate? This is a much farther-fetched idea, and one that has little evidence in support when referring to Atlantic salmon. Much information is required before we can begin to entertain the idea seriously, and data relating to the normal sex ratios of Atlantic salmon would be considered especially important.

The actual sex ratios in populations of mature wild Atlantic salmon are generally between 1:1 and 1:2 in favour of the female element of a given population. However, in some river systems this ratio is as high as 1:4 in favour of females in the returning populations (adults returning from the sea) (Moore, A., CEFAS, personal communication). This could reflect changes in the life-history strategy of the fish; or changes resulting from environmental factors, such as pollution from the agricultural (pesticides and insecticides) or the pharmaceutical (by-products of the contraceptive pill and/or other drugs) industries. Of course, other unknown factors may well be contributing to this shift in the sex ratios. In order to build a comprehensible picture that could explain this imbalance, we need information on the sex-ratios of a given population both before and after migration to sea. This information would be the first step(s) in answering a number of questions. These would include whether up to 80% of outgoing young salmon are in fact female, and if so, the possible reasons for it. Also relevant is whether there is a sex-biased survival mechanism operating at sea, as suggested by Spidle et al. (1998). Equally important, this data may help us to determine whether individuals are undergoing sex-reversal at some stage of their life-

cycle and why. The answers to these questions are of great significance to those with commercial, conservational, and scientific/academic interests, and so it is important that there is a reliable method of identifying the sex-ratios in Atlantic salmon, both before their migration to, and on their return from, the sea.

During the breeding season, close to spawning, it is not difficult to distinguish between adult male (cock) and female (hen) salmon. Both sexes are in their breeding dress – a combination of 'tartan' colours, though the males are generally more coloured than females of similar age. The males also have an enlarged adipose fin, and an elongated head with a hooked protuberance from the lower jaw known as a 'kype', typically used in fighting with other males. Females never develop a kype, and so an animal displaying a kype can be positively identified as a male.

Fig. 1.1.7

Cock (top) and Hen (bottom) Atlantic salmon in breeding dress. Note the enlarged adipose fin, enlarged head and fully developed kype on the cock salmon.

Unfortunately, at earlier stages of the Atlantic salmon life cycle, identification by morphological examination is not possible. The only reliable method of gender determination available is by histological assessment of the gonads, which necessitates sacrificing the individual. This is not acceptable in populations of salmon where numbers are depleted, nor is it a viable option for aquaculture. Even this method has its limitations, as the individuals MUST be of sufficient maturity so that their reproductive organs are developed enough to enable distinction between the male and female gametes.

The challenge that has presented itself is the need for a reliable test for the gender of an individual animal, at any life-stage, without the need for sacrificing it. A genetic test for gender is a very attractive proposition since only small amounts of tissue are required, and therefore it can be done without permanently harming the animal. However, the difficulties encountered here are that the mechanisms of gender determination appear to be much more variable across the animal kingdom than is generally realised.

1.2 Genetic and Other Mechanisms Underlying Gender

The most studied, and invariably the best understood, gender system is the mammalian system, where the development of an embryo into a male or a female is determined by chromosomal complement (XX = female, XY = male). However, it is not this simple across the animal kingdom, and the aim of the following sections is to give a snapshot of some of the differing mechanisms that appear in the various animal groups.

1.2.1 Sexual Differentiation and Sexual Determination

Sexual differentiation is the process by which differences between the male and female forms of a species (where such differences exist) separate during development. The mechanisms of sexual differentiation are by no means uniform in sexual species, in fact far from it. The way differentiation occurs varies from species to species. It is far beyond the scope of this study to explore in detail the mechanisms that exist in invertebrate species (however some examples may be mentioned), and so focus will be concentrated on mechanisms of sexual differentiation and determination in vertebrates.

An important point to highlight here is not to confuse sexual *differentiation* with sexual *determination* – many consider that the two are the same. However, difference between the two definitions does exist. As explained above, sexual differentiation is

the **event** or **sequence of events** that occur when the differences between the sexes begin to propagate. Sexual determination is **when** these events or processes occur, and thus can be used to predict the sex of an individual. For example, the initiation of the development of testes is usually a male *determining* factor, as is the initial event in the development of ova for the female component of a population. The development of the organs themselves from unspecified gonads into testes or ova is known as sexual *differentiation*.

1.2.2 Mammalian Sex

Currently, it is generally accepted that the process of sexual differentiation is a sequence of events in which the establishment of chromosomal sex occurs at the time of fertilisation, as is the role of chromosomes (Uguz et al., 2003). The initial concept of chromosomal involvement in sexual differentiation was suggested by Morgan (1914), who conducted numerous studies on fruit flies (*Drosophila melanogaster*) to understand the mechanism of sexual differentiation. These studies suggested that the number of X chromosomes present determined the sex of an individual – flies with a single X (variants XY or XO) were observed to become males, whilst flies with two or more X chromosomes (XX, XXX, XXY) were observed to become females (reviewed by Vogel and Motulsky, 1979). And so the significance of the Y chromosome was originally thought to be merely for the fertility of the male (Stern, 1957). Moving on from these early observations, the popular belief of the role (or lack thereof) that the Y chromosome plays in vertebrate sex differentiation and determination has changed as

a result of the development and application of new techniques for karyotyping chromosomes.

The mechanisms governing sexual differentiation in mammalian species have been extensively studied and are generally widely accepted. Humans and other mammals operate by an XX:XY chromosomal sex determination system. Females are determined by the homogametic XX, whereas males exhibit heterogamety (XY). Despite the findings of Morgan (1914) as outlined above, the currently accepted view is that the sheer presence of the Y chromosome, regardless of the number of X chromosomes present, determines 'maleness'.

1.2.2.i The Y Chromosome

Although it is evident that the control of sexual differentiation is highly dependent on the genetic information encoded in the Y-chromosome (Merchant-Larios and Moreno-Mendoza, 2001; Salas-Cortes et al., 2001; Ohe et al., 2002), the exact molecular mechanisms are still yet to be fully understood. Mammalian X and Y chromosomes differ significantly in size and gene content. The X maintains the same 5% of the haploid genome and the same suite of genes in all *eutherian* (placental) mammals (Graves, 2002). Most of the ~1500 genes found on the 165-Mb human X chromosome have housekeeping or specialised functions in both sexes (International Human Genome Sequencing Consortium, 2001; Venter, et al, 2001). In stark contrast, the Y chromosome is much smaller and is almost devoid of genes, and the relatively minute – c.50 functional genes on the 60-Mb human Y are embedded in a quagmire of

repetitive sequence DNA (www.ncbi.nlm.nih.gov/genome/guide/human). At least half of these Y-based genes are specialised for sex and spermatogenesis, though curiously, their presence/absence, copy number, sequence and activity vary between species.

Comparative studies between the three major mammal groups, *eutherians* (placental, e.g. humans), *metatherians* (marsupials – often but not always pouched, e.g., kangaroo), and *monotremes* (egg-laying, e.g. platypus, echidna) has shown that sex chromosomes consist of two regions of distinct origins (Graves, 1995). One region on the X chromosome is conserved in all mammals and so must be ancient. However, another region is on the X in eutherians but autosomal in other mammals, suggesting that it must have appeared recently in the eutherian lineage. This region, making up most of the short arm of the human X, exhibits signs of its recent appearance as many of its genes do not undergo *X inactivation* – stable and heritable epigenetic silencing of one X chromosome in the somatic cells of female mammals. In the Y chromosome, there also exists a region that is conserved in all mammals, and an additional region that is only present in eutherians (Waters et al., 2001). For the purposes of understanding the evolutional history of the mammalian sex chromosomes, the observation that the ancient part of the sex chromosomes is shared between all the major mammalian groups means that the mammalian Y chromosome must have appeared before monotremes diverged from the eutherians and metatherians about 170 million years ago.

Despite the significant differences in size and gene content, there is considerable homology between the mammalian X and Y chromosomes, which goes some way to support a hypothesis that mammalian sex chromosomes evolved from an autosomal

pair (Graves & Shetty, 2001). This hypothesis was first advocated by Susumo Ohno (1967), and was developed to explain nuances in sex chromosome differentiation in different snake families. The hypothesis suggests that a pair of autosomes became proto-sex chromosomes when a new sex-determining allele evolved on one of the pair. Other alleles with functions in that sex subsequently accumulated near the new 'sex-determining locus'. This now meant that suppressing recombination between the proto-sex chromosomes became favourable, allowing inheritance of the group as a sex-specific region. Consequently, further accumulation led to further suppression of recombination. Within this non-recombining region, mutations, deletions, insertions and amplification led to gene inactivation and the accumulation of repetitive elements. This accumulation has been subject to debate over the course of decades, and among the arguments are the proposals known as "Müller's ratchet" – the elimination of Y chromosomes with the fewest mutations from a given population, and the "hitchhiker" hypothesis – selection of Y chromosomes based on favourable mutations. As a result, mutations or deletions that are on the selected Y subsequently "hitchhike" to fixation (Charlesworth, 1991). And so it seems that the acquisition of a sex-determining allele by the Y chromosome triggered an ongoing process of Y chromosome degradation.

When considering the cascade of events as described above, we are assuming that an initiating event defined the proto-Y chromosome, and that this event was the acquisition of a testis determining factor (TDF). Next was the accumulation of other male-specific genes, leading to suppression of recombination and subsequently the degradation of the Y chromosome. The obvious question marks here point at the TDF – when and how did it become acquired? In search of the answers, comparisons must be made between the sex-determining systems of mammals and the other vertebrates.

Solari (1993) points out that there is a diverse selection of chromosomal, genetic and environmental sex-determining systems, and that despite this diversity, there is no obvious homology to the mammalian system. Taking birds and snakes together, there is similarity with mammals in that they have distinguishable sex chromosomes, but unlike mammals it is the females that have the heterogametic ZW, and the males that have the homogametic ZZ pair of chromosomes. Comparative gene mapping has shown that there is no relationship between the mammal XY and the bird ZW pairs (Nanda et al., 1999), which suggests that they evolved independently from different autosomal pairs. This in turn suggests that the mammalian Y chromosome evolved *after* the branching of mammals from reptiles 310 million years ago. In summary, the emergence of the Y chromosome came about, at the very latest, *before* the branching of monotremes from eutherians and marsupials; and at the very earliest, *after* the branching of mammals from reptiles. This puts the age, or the appearance of, the Y chromosome at somewhere between 170-130 million years ago (Mya).

When considering how the TDF was acquired by the mammalian Y chromosome, we must go back to Ohno's hypothesis, which predicts that the genes on the Y chromosome are relics of genes that were on the ancient proto-sex chromosome or the added region. If this is the case, then all the genes on the Y should have X-borne homologues – that is, equivalent genes on the X chromosome – whose sequence and function have remained generally unchanged. The genes on the mammalian Y chromosome are classified into two groups. Class I genes are single-copy, and have homologues on the X chromosome. 'Class II' genes appear to be testis-specific and have no known homologue on the X chromosome. They are, in effect, male-linked

genes that seem to have been acquired by the proto-Y and amplified (Lahn & Page, 1997).

However, homologues have been found on the X chromosome for some of these genes considered to be multi-copy and testis-specific, for example *RBMY*, a candidate gene for spermatogenesis (Delbridge, 1999) has a homologue on the X chromosome, *RBMX*. The notion of two discrete classes of Y-borne genes is weakened further when other species are considered, because several genes belonging to Class I in humans have been found to be Class II in rodents, for example *ZFY* (Koopman et al., 1991). Based on this evidence, it appears that many Y-borne genes exhibit a broad spectrum of degradation and specialisation – from full homology to complete loss. Class I genes are simply those at the beginning of the process. Some Class II genes have been acquired from autosomes, but many descended from genes on the proto-XY and evolved to have a selectable male-specific function (Graves, 2001). Many of these male-specific genes were amplified in the race to stay ahead of inexorable degradation, and have survived – at least until now – but could inevitably be superseded. For example, a putative spermatogenesis gene *UBE1Y* is Y-borne in mice and marsupials but not in primates (Mitchell et al., 1998).

Another example of degradation and specialisation – where genes with wide-ranging functions have been metamorphosed into male-specific (and possibly testis-differentiating) genes on the Y chromosome, apart from the previously mentioned *RBMY* with its X chromosome homologue *RBMX*, is the marsupial *ATRY* gene (testis-specific), which has a ubiquitously expressed X-borne homologue *ATRX* (Pask et al., 2000).

And so the evolutionary progression of the chromosomes in mammals has meant that sexual differentiation is in effect determined by the presence or absence of the specialised Y-chromosome. Aside from cases where gene mutations have caused an alteration in the sexual differentiation cascade in mammals, plasticity within the system is not generally considered an issue.

1.2.3 Fish Sex

With around 25 000 known species, this is by far the largest group of vertebrates. The variety of physical and behavioural characteristics that have evolved and been adapted for survival in the greatly differing aquatic habitats of this planet is truly extraordinary. This variety extends even, to the way sexual determination occurs in the different species. This section explores some of these variations, and puts into perspective what a complex world sexual determination and differentiation actually is in fish.

1.2.3.i Hermaphroditism (Inter-sexuality) in Fish

Hermaphroditism in fish is not uncommon, and many species naturally have hermaphroditic characteristics at least at one stage or another in their life cycle. Unlike other vertebrates, fish (depending on the species) can be either gonochoristic or hermaphrodite. Gonochorism is where the male and female sexes are represented by

two *different* individuals. Therefore, gonochoristic fish are those that will develop only as males, or only as females, and will remain as that gender throughout their lives. However, it is important to note that the sexually mature state that an individual expresses is not necessarily a reflection of the *initial* gonadal developmental pathway. A gonochoristic fish may not develop directly into a male or female; they may develop with gonads that are initially hermaphroditic, and subsequently resolve into fully functional testes or ovaries exclusively (Devlin & Nagahama, 2002). There are two major types of gonochorists, as identified by Yamamoto (1969): first, the *differentiated* gonochoristic species, where early gonad development advances from a 'pre-gonad', before differentiating into either ovaries or testes. An example of this kind of species is the Coho salmon, *Oncorhynchus kisutch* (Piferrer & Donaldson, 1989). Alternatively, there are *undifferentiated* species, where all individuals initially develop ovarian tissue, before in approximately half the population, the ovarian tissue degenerates, and the gonad is flooded with additional somatic cells. Development then proceeds to form an initially intersexual gonad, and finally resolving into a normal testis. Such a species is the zebrafish, *Danio rerio* (Takahashi and Shimizu, 1983). Although these types of fish show what can be referred to as hermaphroditism at some stage in their life cycle, they cannot be classified as a 'normal hermaphrodite'.

Normal hermaphrodites can produce both male *and* female, fully functional mature gametes at one stage or another in their lives (Devlin & Nagahama, 2002), and so it has been an interesting and popular group to study sex determination among vertebrates. There are different classes still, of 'normal' hermaphrodites, each with their subtly different forms of hermaphroditism, although all coming under the collective term – functional hermaphrodites.

‘Synchronous hermaphrodites’ (also called simultaneous hermaphrodites) produce both male and female gametes at the same time – some species can alternate between sperm and egg delivery, for example the Serrano, *Serranus fasciatus* (Peterson, 1990). Some species even have the remarkable ability of internal self-fertilisation (Soto et al., 1992). The second group are the ‘sequential hermaphrodites’. These produce one gamete type, and then sex reverse and produce the other type in an ensuing spawning cycle (Sadovy and Shapiro, 1987). These can be classified into two further types: Protandrous hermaphrodites if they mature as a male first, and then subsequently as a female, with the ovaries taking the place of the testes. Species that mature as females, then as males are referred to as protogynous hermaphrodites (Devlin & Nagahama, 2002). The sex changes in hermaphrodites can be triggered by age, temperature, social or other unknown factors. The most extreme case of hermaphroditism found so far is in the cyprinodont, *Rivulus marmoratus* – a self-fertilizing simultaneous hermaphrodite, not only credited with being the only fish, but also being the only known vertebrate of its kind (Scott Taylor, 2000).

Examples of hermaphroditism or spontaneous sex reversal are very rare in gonochoristic species (Atz, 1964). Such individuals are called abnormal hermaphrodites or intersexes, and are usually observed in the field or laboratory surveys where gonadal development or sex ratio is being studied. Such species include the brown trout, *Salmo trutta*, where an individual was observed producing functional eggs and sperm (O’Farrell & Pierce, 1989). Another is *Oncorhynchus keta*, where four individuals have been identified containing both testicular and ovarian tissue (Devlin and Nagahama, 2002). These results are particularly relevant to the present study,

because these species are related to the Atlantic salmon (*Salmo salar*). Although there are no recorded cases for abnormal hermaphroditism in Atlantic salmon, the occurrence of it in *Oncorhynchus*, and particularly in *Salmo trutta*, means that we must not discard the possibility of it happening undetected.

1.2.3.ii Hormones and Fish

We have established already that fish exist as gonochoristic and hermaphroditic species (see above). While hermaphrodite species change between genders through the course of their natural life cycle, gonochoristic species, as a general rule, remain faithful to their genotypic genders once sexual maturity has been reached. However, stimulation by certain 'outside influences' or more specifically, factors in the environment they are residing in, can cause unnatural sex reversal. Although this extreme is possible, the magnitude of the effects of these factors do differ from species to species (Baroiller et al, 1999). The most common form of environmentally linked sex reversal is through endocrine disruption (discussed below).

There is evidence for hormonal induction of sex reversal in many species, including Atlantic salmon (Pandian & Sheela, 1995). It is possible, through the use of exogenous steroids, to artificially induce sex reversal in many fish species. Reasons for the requirement of sex reversal of fish may vary, depending on its use. For instance, the males of most ornamental fish are more colourful than the females, and so have a higher commercial value than their female counterparts (Pandian & Sheela, 1995). On the other side of the coin, however, females are regarded as being of higher

commercial value as food fish, because they are generally fatter, and more desirable for the consumer. As a result, there are protocols available for both masculinisation and feminisation in a wide range of species. And so, the use of steroids for masculinisation and feminisation (androgens and oestrogens respectively) of fishes is now very common in aquaculture. Aquaculture production has increased very rapidly over the last three decades, so much so that by the mid-nineties – from just 3.9% in 1970 (FAO, 2009) – it accounted for well over 20% of the world's production of aquatic foods of marine and freshwater origin (Donaldson, 1996), and 36% by 2006 (FAO, 2009). In fact, in 2006, aquaculture accounted for 47% of the world's fish food supply. For this reason, sex-reversal has been introduced for use with Atlantic salmon as a food fish, for they do not change sex naturally. It is through this commercial interest in female salmon that production of female-only populations within fish farms by way of hormone induced sex reversal has come about.

The first generation of techniques for hormonal sex reversal were developed in the 1930s by Padao (1937). These involved the administration of pituitary glands from mature individuals homogenised in physiological saline (Donaldson & Hunter, 1983). Second-generation methods involved the use of acetone-dried homologous or heterologous fish pituitary glands, partially purified from fish gonadotropins and human chorionic gonadotropin (hCG) (Donaldson, 1996). Current methods for direct sex reversal utilise natural oestrogen (estradiol-17β) for feminisation, and synthetic 17α-methyltestosterone for masculinisation (Pandian & Sheela, 1995) during the developmental stage of production fish. Current indirect techniques involve hormonal, genetic or environmental manipulation in the previous generation to produce monosex gametes. Monosex female salmonids have been produced by this method of indirect

feminisation (Donaldson & Devlin, 1996) and sterile (monosex female triploid) salmonids have been produced by indirect feminisation plus pressure or temperature shock treatment to induce triploidy (Piferrer et al., 1994).

The steroids are usually administered in the food given to the fish, but sex reversal can also be achieved without using food to administer the steroid. Piferrer and Donaldson (1992) successfully yielded 100% females in *Oncorhynchus tshawytscha*, using a single immersion for 8hrs per day in 400μg estradiol-17β per litre of living water for 35 days. Thus there is evidence that natural and unnatural steroids present in the environment can affect salmonid gender. This revelation is particularly interesting in the context of Atlantic salmon sex ratios, because it represents the possibility of a shift in the ratios as a result of human-generated hormone pollution. However, for an extensive study into the true effects (if any) of human-generated homone pollution in salmonid populations and more specifically, Atlantic salmon populations in the natural environment, we need to understand the mechanisms that determine sex in Atlantic salmon in the first place, and be able to assess the sex ratios at various life stages in order to quantify any effects that may be uncovered.

1.2.3.iii Genetics of Sex in Fish

There is enormous variety in the chromosomal mechanisms of sexual differentiation in fish, perhaps unsurprising given the vast number of species in the group. Both of the major gonosomal systems (XX:XY and ZZ:ZW) of sex determination found in other non-mammalian vertebrates are represented. For instance, *Tilapia mossambicus*

and *Tilapia nilotica* are examples of the homogametic female (XX) and heterogametic male (XY) system; and *Tilapia hornorum* and *Thorichthys aureus* are examples of the homogametic male (ZZ) and heterogametic female (ZY) system (Chen, 1969). This highlights the fact that chromosomal systems of sexual determination can vary to a high degree - even among closely related species. In turn, this indicates that the chromosomal mechanisms of sex determination in fish are far more complex than in other vertebrates. One view is that there are polygenic mechanisms of sex determination in fish (Bull, 1983), meaning that the sexual determination factors or genes are distributed over several loci. These so-called polygenes involved in sex determination in fish include genes or regulatory elements in the X, Y, W and Z chromosomes, and also in the autosomes, epistatic genes, and possibly some other unknown factors (reviewed by Solari, 1994).

Even more complex systems where there are multiple sex chromosomes in existence in a population have also been described. One of the best studied species is the popular tropical aquarium fish, the Platyfish, *Xiphophorus maculatus*, where three types of sex chromosomes, X, W and Y, coexist in a population. Gender is dependent on the chromosomal complement: WX, XX and WY fish become females, whereas XY and YY fish become males (Volff, 2001). Another example of a multiple system is the $X_1X_1X_2X_2/X_1X_2Y$ system, found in Mexican populations of Cyprinodontidae (Uyeno, 1977).

Various techniques are employed by those studying the mechanisms of sex in fish, ranging from simple cytogenetic analysis, to the isolation of sex-specific DNA markers. Depending on the species, each of these techniques have had varying degrees

of success in elucidating the mechanisms by which each species determines and subsequently, differentiates sex.

The simplest of these techniques is cytogenetic study to determine the karyotype of male and female individuals within species. If heteromorphic chromosomes are found, the mechanism of sex determination can be reasonably regarded as being genetic. Others include analysing the sex ratios among families; examining the progeny sex ratios from sex-reversed individuals; and development of monosex strains of species (for review, see Devlin & Nagahama, 2002). One area of study that has seen significant progress in recent years is the quest to isolate sex-specific DNA markers within the genome of target species. One of the biggest obstacles to this progress is the sheer amount of genetic information that needs to be examined in order to successfully isolate a sex-specific DNA marker (Devlin and Nagahama, 2002). Fortunately, the continual development of molecular biology techniques has made it increasingly possible to reveal sequences that are present in one genome but absent in another. It is now possible to compare fragment patterns from males and females to identify any sex-specific differences in species (but not all).

Fluorescence in situ hybridisation (FISH) is a tool used to detect and to localise the presence (or absence) of specific sequences of DNA on chromosomes. It makes use of *fluorescent probes* – DNA sequences (specifically, those of interest) tagged with fluorescent molecular markers, that when denatured to single-stranded DNA, can hybridise with target DNA (the DNA to be tested) where it is complementary to the probe sequence. The resultant hybrid can then be analysed using fluorescence microscopy, where the fluorescing tagged sequences can be identified, and therefore

their location in the DNA determined. In model species, the technique has been used to chart the localisation of centromeric repetitive sequences – both of a highly repetitive and a moderately repetitive nature; probes have also been developed that are specific for whole genomes, whole chromosomes, and chromosome subregions in some species; sex-specific sequences have also been mapped and localised in several species, using FISH (reviewed in Phillips, 2001). In the context of the current study, the sequences of interest would be those that have been identified as being sex-specific.

Genes that are believed to be sex-linked in other vertebrates (such as *H-Y antigen*, *ZFY*, *Dmrt1*) have also been found in fish, but although they may play a role in sex determination and differentiation, they are more likely a consequential, rather than causal effect of sex determination (Devlin, 2002).

BKm is the term used for the GATA-GACA repetitive (minisatellite) sequences that were first isolated from a female banded krait snake (Singh et al., 1980). These sequences have been found to hybridise extensively to the W chromosome of female snakes (females being the heterochromatic sex) (Singh et al., 1980), and is also very well conserved among eukaryotes. It has also been reported to be sex linked in many species (Jones & Singh, 1985; Epplen, 1988). Tandem repeats of the sequences cross-hybridise with the sex chromosomes across a wide variety of evolutionary branches; from the W chromosome of birds (Jones and Singh, 1985), to the X chromosome of *Drosophila melanogaster* (Singh et al., 1981), to the Y chromosome of the mouse (Epplen et al., 1982). A study by Lloyd et al. (1989) hybridised *BKm* sequences to Rainbow trout (*Oncorhynchus mykiss*) DNA, and found that although there was

polymorphism of the sequences, no sex-specific differences were apparent. *BKm* based sex-specific sequences have also not been found in the Channel catfish (Tiersch et al., 1992), nor in the Turbot or the Atlantic salmon (Husebye et al., 1994).

ZFY is a gene closely linked to the human *Sry* gene. It was initially thought of as a candidate testis determining factor (TDF) before the isolation and identification of *Sry* (Sinclair et al., 1990; Koopman et al., 1991) as the male determining master gene in mammals. There is an X-chromosome homologue of *ZFY*, called *ZFX*, which is widely conserved throughout evolution. The reason why these genes are of such interest is that there exists orthologs of *ZFY* and *ZFX* in all groups of vertebrates, including fish (Poloumienko, 2004). However, despite many years of relevant research, the physiological role(s) of *ZFY* and *ZFX* remain unclear.

ZFY probes have been found to hybridise to sequences found on the Y-chromosome of every eutherian mammal that has been tested, suggesting that it is specifically Y-linked (Page et al., 1987). PCR tests on the Channel catfish (*Ictalurus punctatus*) revealed that *ZFY*-specific primers did not generate amplified fragments from genomic catfish DNA (Tiersch et al., 1992). The use of *ZFY* probes on Turbot (*Scophthalmus maximus*) revealed hybridisation in both male and female individuals (Husebye et al., 1994), which is consistent with the results of work done on Rainbow trout (*Oncorhynchus mykiss*) and Sturgeon (*Acipenser transmontanus*) (Ferreiro et al., 1989). These probes also hybridised with genomic DNA from Atlantic salmon, but again no sex-related differences were identified (Husebye et al., 1994).

In the Channel catfish study (Tiersch et al., 1992), primers that specified a region shared completely by *ZFY* and *ZFX* did yield amplified fragments. This correlates to previous work on mammals indicating that sequences corresponding to *ZFX* are more conserved than sequences corresponding to *ZFY* (Page et al., 1987). This would appear to be in agreement with the previously mentioned "Ohno's Law", which proposes that the X and Y chromosomes diverged from an ancestral autosomal pair. Ohno's hypothesis also suggests that the Y-chromosome has lost a lot of its genetic material to become a specialised sex determinant, whereas the genes on the X-chromosome have essentially remained conserved throughout mammalian evolution. The fact that *ZFY* homologues are a lot less conserved than that of homologues of *ZFX* may indicate that *ZFY* has recently differentiated from the *ZFX* gene. Applying this logic further suggests that the X and Y chromosomes in fish – at least in the species described here – are at the earlier stages of differentiation, and that this varies somewhat between species.

Dmrt1bY (also known as *DMY*) was identified by linkage mapping of the sex chromosomes of the Medaka fish, *Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002), and found to be Y-chromosome specific. The gene encodes a putative transcription factor, and is part of a family of genes which share a DNA-binding domain called the DM-domain (Zhu et al., 2000). It is highly conserved during evolution, and its mammalian homologue is thought to be involved in sex determination as part of the downstream genetic cascade of events. For example, deletion of a copy of *Dmrt1* in humans is associated with XY male to female reversal (Raymond et al., 1999). The identification of this gene as a 'master regulator' sex in the Medaka fish raised the question of whether it might serve a similar role in other

fish. Investigation on several species, both closely related to, and more distantly related species by Kondo et al. (2003) revealed that *Dmrt1bY* was absent in *Oryzias celebensis* and *Oryzias mekongensis* (same genus), and also in the Guppy, Tilapia, and Zebrafish (more distant). The absence of the gene from all other species assessed so far would indicate that it has recently appeared in a single branch of the fish phylogenetic tree – that of *Oryzias latipes*.

It is generally accepted that Salmonids operate an XX:XY chromosomal system, with males being the heterogametic sex, however few species have been found to have clearly morphologically differentiated sex chromosomes (Phillips & Ráb, 2001). Among the species most widely studied are those of the genera *Oncorhynchus* and *Salvelinus*, and these studies are explored in greater detail later. Suffice to say, however, the location of the sex determining locus in *Oncorhynchus* species has long since been identified (Woram et al., 2003) and much work has been undertaken, in attempts to elucidate genetic markers relating to sex. There are comparatively fewer studies involving the *Salmo* species, and it was much more recently that the sex determining locus in Atlantic salmon was even identified (Artieri et al., 2006). An extensive study using randomly amplified polymorphic DNAs (RAPDs) to screen for sex-linked genetic markers failed to detect any such entities (McGowan and Davidson, 1998), despite the approach having been successful when attempted with plants (Hormaza et al., 1994), birds (Lessels and Mateman, 1998), and other Salmonids (Devlin et al., 1991; Du et al., 1993; Forbes et al., 1994).

1.3 *Sry* and the *Sox* gene family

In the early 1990's, significant breakthroughs were made in the quest for understanding the mechanisms of sexual determination and differentiation in vertebrates. The cell types and more specifically, the gene responsible for male sex determination in mammals were conclusively identified. Palmer and Burgoyne (1991) showed that only Sertoli cells exhibit a selective bias for the presence of a Y chromosome. It was also shown in the same year that the Y chromosome gene, *Sry* (*Sex-determining Region on Y*) could direct male development in a female mouse carrying an *Sry* transgene (Koopman, 1991). The two discoveries were linked later by Hacker et al. (1995), who showed that *SRY* is expressed at the same time as male sex-determination in pre-Sertoli cells.

In this chapter, we discuss *Sry* and the *Sox* genes, and their roles in different vertebrate groups.

1.3.1 *Sry*

The human *Sry* gene encodes a transcription factor that is believed to activate and/or repress target genes (Sánchez-Moreno et al., 2008), the ultimate influence of which is the differentiation of the indifferent gonad into testis-specific cell types, and organises into testis-specific morphology. The encoded *SRY* protein is a 204 amino acid nuclear protein containing a region of 79 amino acids that shares homology with the HMG

(high mobility group) proteins (Sekido, 2010). This group of proteins share what is known as an 'HMG box' domain, which binds to DNA at specific target sequences and bends it through specific angles. This brings about interactions between DNA sequences that are non-adjacent, and would therefore not normally have an effect on one another (Graves, 2002). This property makes them ideal for coordinating DNA structure, which in turn would suggest that the *SRY* protein may act more as an architectural factor that helps to coordinate local chromatin structure, rather than as a classical activator – or repressor – of gene activity.

Experimental evidence has strongly pointed towards *SRY* as the most likely testis determining factor (TDF) in eutherian and metatherian mammals (Salas-Cortes et al., 2001; Ohe et al., 2002). Analysis of *SRY* mutants isolated from human XY Swyer syndrome individuals has revealed that most of the mutations lie within the HMG box, and the resulting products of the mutant proteins either bind to DNA poorly, or bend it through the wrong angle (Graves, 2002). This suggests that much, if not all, of the activity of *SRY* is dependent on its capacity to bind and bend DNA in the appropriate manner, effecting chromatin structure to subsequently influence the activity of an as yet unknown target gene. In summary, it is thought that the product of the *Sry* gene binds DNA at a specific target site (AACAAT) and bends it through a certain angle (Harley et al., 1992), perhaps bringing sequences (or bound proteins in the vicinity of the target site) together, and causing changes in chromatin structure and gene activity.

Although the structure of *SRY* differs between mammalian species, its TDF activity appears to be conserved. Lovell-Badge et al. (2002) showed that human *SRY* can still induce testis development in XX transgenic mice when it is expressed under the

control of mouse regulatory sequences; and a goat BAC DNA containing *SRY* also leads to testis development in the mouse (Pannetier et al., 2006). It has been shown that mutations in the *Sry* gene lead to female development in XY mice, and when expressed as a transgene in XX embryos, *SRY* alone is sufficient to induce testis development (Koopman et al., 1991). As such, it is believed to be the only Y-linked gene required for testis – and therefore, male – development in mammals (Canning & Lovell-Badge, 2002).

However, the exact mechanisms involving *SRY* as the initiating factor for testis formation are still yet to be disseminated, though Koopman et al. (1990) suggest that inactivation of the anti-müllerian hormone (*AMH*) gene is involved; and Ohe et al. (2002) reported that the *Sox* genes (discussed later) regulate the biological function of the *Sry*, *Sox6* and *Sox9* gene products. Recent work has suggested that *SRY* binds to a testis-specific enhancer of *Sox9*, and activates *Sox9* expression in co-operation with steroidogenic factor 1 (*SF1*) to promote Sertoli cell differentiation in mice (Sekido & Lovell-Badge, 2008). Even more recently, it has been suggested that the cerebellin 4 precursor gene (*Cbln4*), encoding a transmembrane protein containing a signal peptide, which may be cleaved and serve as a secreted molecule may also be a direct target of mouse *SRY* (Bradford et al., 2009). However, the biological function of this secreted molecule in testis differentiation is not known.

Whatever the mechanism(s) involved, it seems that timing is of crucial importance for testis differentiation (Hiramatsu et al., 2009). Sex reversal assays using transgenic mice carrying a heat-inducible *Sry* allele revealed that *SRY* triggers upregulation of *Sox9* expression and in doing so, initiates testis differentiation in XX gonads between

11.0 and 11.25 days post coitum (dpc). Inducing *Sry* expression at 11.5 dpc still produced initial *Sox9* upregulation, but this was not maintained and eventually led to impairment of downstream events. When inducing *Sry* expression beyond 11.5 dpc, *Sox9* upregulation was not detected. These observations may be explained by the activation of the ovarian pathway in XX gonads from 11.5 dpc onwards, which in turn would suggest that the delayed expression of the *Sry* transgene is unable to overcome the ovarian pathway (Sekido, 2010).

In summary, despite being isolated in 1990 and confirmed as the mammalian TDF shortly after, the molecular actions of *SRY* are still yet to be proven. However, we have still learned much about this remarkable gene. We now know that the *SRY* protein is poorly conserved outside of the HMG box domain among mammals (Sekido, 2010), but its TDF function appears to be well conserved. Also apparent is that *SRY* co-operates with SF1 and activates *Sox9* expression (but we don't know how), and this in turn leads to testis differentiation in the bipotential gonad. The capacity of *SRY* to initiate testis development is also subject to a limited window of time post-conception. Whilst arguably the most important discovery so far in the journey towards understanding the mechanisms of gender determination in mammals, there is clearly much to learn regarding how *Sry* itself is regulated, and how it interacts with downstream genes to initiate male development.

1.3.2 The *Sox* Gene Family

Characteristic of the *SRY* protein is the HMG-box domain. The HMG-box domain itself is an extremely versatile protein domain that facilitates the DNA binding of both sequence specific and non-sequence specific proteins (Štros et al., 2007). Any protein containing the HMG-box domain is a member of one of three classes of a diverse and very large 'superfamily' of high-mobility-group (HMG) proteins. Unfortunately it is beyond the scope of this thesis to explore these proteins in detail, and so the interested reader is directed to an excellent review by Štros et al. (2007).

As has already been discussed, the *Sry* gene encodes a protein that contains the HMG-box domain. We have also established that there are many proteins that contain the HMG-box, and even more that are part of the HMG protein 'superfamily'. A number of these HMG-box containing proteins share close homology with the HMG-box domain found in *SRY*, and these are what have been termed the '*SRY*-like, HMG-box-containing' (*Sox*) proteins, each encoded by a gene with the corresponding name.

1.3.3 The *Sox* Gene Groups

Since the discovery of *Sry* in 1990, a total of twenty vertebrate *Sox* genes have been characterised, and these have been categorised into eight groups (A-H), including two sub-groups (B1 and B2) (Lefebvre et al., 2007).

Gene	*Sox* Group
Sry	A
Sox1	B1
Sox2	B1
Sox3	B1
Sox4	C
Sox5	D
Sox6	D
Sox7	F
Sox8	E
Sox9	E
Sox10	E
Sox11	C
Sox12	C
Sox13	D
Sox14	B2
Sox15	G
Sox17	F
Sox18	F
Sox21	B2
Sox30	H

Fig. 1.3.1

The 20 vertebrate *Sox* genes, and their designated groups (adapted from Lefebvre et al. 2007).

Wright et al. (1993) were the first to propose that the *Sox* family of genes could be further categorised into groups, designated A-F, on the basis of how their primary sequences and other structural indicators (such as intron-exon organisation) compared to each other. *Sox* sequences belonging to the same group share a high degree of homology (generally between 70-95%) both within and outside of the HMG box, whilst those from different groups generally share partial homology (≥46%) within the HMG box and none outside of it (Lefebvre et al., 2007).

The original six groups were then further expanded to seven groups after work done by van de Wetering & Clevers (1993); and Meyer *et al.* (1996) in the identification of *Sox15* and human *Sox20*; and then to eight, after a study by Osaki et al. (1999), in identifying *Sox30*. Human *Sox20* has since been reclassified as human *Sox15* (Ito, 2010), although this does not affect the configuration of the *Sox* groups.

1.3.4 The *Sox* Genes in the Invertebrates

It is beyond the scope of this thesis to explore in detail all of the knowledge we have of the *Sox* genes in invertebrates. However it is perhaps useful to outline the 'headline findings' from the field. None of the *Sox* genes so far characterised in invertebrates have been implicated in sex determination but (some) have important developmental functions in the invertebrate model systems *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Strongylocentrotus purpuratus* (Phochanukul & Russell, 2010).

Three or four discernable *Sox* genes have been found in the basal metazoans (sponges and the placozoan *Trichoplax*), with at least one clear Group B member, and single representatives of the C/D, and E/F groups (Larroux et al., 2008). In the Cnidarians and Ctenophorans (anemones, corals, jellyfish and comb jellies), also collectively referred to as the Radiata, it is apparent that there is a significant increase in the number of *Sox* genes present in the genomes. For example, 14 genes in the scarlet sea anemone, *Nematostella vectensis*, and 12 genes in a freshwater hydra, *Hydra magnipapillata* (Putnam et al., 2007); 10 genes in a sea hydra, *Clytia hemisphaerica* (Jager et al., 2006); and 13 genes in a comb jelly, *Pleurobrachia pileus* (Jager et al,

2008) among others. Lineage-specific duplication and divergence is thought to account for this expansion in *Sox* gene numbers in these species, as a number of the *Sox* sequences are not easily classified into the established groups present in higher animals (Larroux et al., 2008). Interestingly, there are fewer *Sox* genes in the Protostomes than there are in the Radiata – to date, less than ten genes have been found in all species examined (Phochanukul & Russell, 2010). In contrast to the case in protostomes, the Chordate widely regarded as an archetype for the predecessor of the vertebrates, the lancelet (*Branchiostoma floridae*) has at least 13 *Sox* genes, with at least one representative in each of the Groups B-F (Putnam et al., 2008). Taking all of this into consideration, it would appear that the *Sox* family of genes began to diversify in the metazoan stem branch, prior to the divergence of the demosponges, and further diversification occurred later in the ctenophores, cnidarians, and vertebrates. In contrast, however, *Sox* gene *loss* seems to have occurred in the protostomes (Jager et al., 2006).

In insects, the most studied group of organisms in relation to the *Sox* genes are the Drosophilids. Early work indicated that there are eight *Sox* genes encoded in the genome of *Drosophila melanogaster* – consisting of four Group B genes and one each from Groups C-F (Cremazy et al., 2001). More recently, following the sequencing of an additional eleven *Drosophila* species, it seems that the complement of eight *Sox* genes found in *Drosophila melanogaster* is conserved among all flies (Clark et al., 2007).

As data on insect genomes has emerged, a remarkable degree of conservation in the *Sox* genes has been revealed. For example, the mosquito *Anopheles gambiae*, which

shared a common ancestor with *Drosophila* 250 million years ago, has an identical complement of *Sox* genes; the hymenoptera (comprising bees, wasps, and ants) have an additional Group E gene (Phochanukul & Russell, 2010); and the flour beetle, *Tribolium castaneum* has an additional Group B gene in comparison with *Drosophila* (Richards et al., 2008). The additional genes seen in the hymenoptera and the beetle probably represent lineage-specific duplications or expansion, as the structure and genomic organisation of the *Sox* gene family appear to be very similar across the sequenced insects (Wilson & Dearden, 2008). As discussed by Phochanukul & Russell (2010), it will be interesting to see if the 'core' complement of *Sox* genes so far discussed is maintained outside of the holometabolous insects – insects where development consists of four life stages: embryo, larva, pupa, imago (adult) – and whether this extends even further to the arthropods.

Much of the available literature, especially works detailing functional studies in the *Sox* genes in invertebrates appears to focus on *Drosophila* species, presumably due to *Drosophila's* status as model species. Despite this, the (structural) sequence relationships between all of the invertebrate *Sox* genes appear to be relatively straightforward, and there is a high degree of conservation between species. However, homology in function is far more confused, especially in the insects. For example, although functional data is only available for *Drosophila Sox* genes, the expression data from *Apis mellifera* (honeybee) shows very little conservation. What is clear, however, is that the *Sox* suite of genes have important roles at different stages of development – certainly in *Drosophila* (Phochanukul & Russell, 2010), and given the sequence homology among the different invertebrate *Sox* genes, there are as yet no reasons to believe that they do not have similar roles in the other invertebrate groups.

The study of *Sox* genes in the invertebrates is becoming increasingly tractable, and many invertebrate models are more amenable to functional studies than before, with the advent of methods such as RNAi-mediated gene knockdown. As more work is completed in the field, our understanding of the functional roles of Sox proteins in different invertebrates will increase dramatically.

1.3.5 The *Sox* Genes in Vertebrates

Much more work has been done on the functions of the *Sox* genes in vertebrates. In this section, each of the *Sox* gene groups will be discussed in turn, exploring their known functions in vertebrates, and where possible, emphasis will be placed on function in fish.

1.3.5.i *Sox* Group B1

The Group B1 *Sox* genes, comprising *Sox1*, *Sox2* and *Sox3*, contain transcriptional activation domains (Bowles et al., 2000), and are implicated in early development and neurogenesis in many species. Members of this gene group control the expression of distinct sets of genes in a cell-specific manner by interacting with specific partners (Kamachi et al., 2000; Miyagi et al., 2008).

The expression of *Sox2* in the developing CNS across all of the vertebrates is very conserved. This was the first characteristic of *Sox2* to attract attention, before interest in its many functions began to increase (Collignon et al., 2006).

In the mouse, *Sox2* is first expressed in early embryos, in the epiblast, the extra embryonic ectoderm, and the chorion, then later in the neural precursors, the ependyma, the neuron, and the thalamus, and absence of *Sox2* is lethal to the embryo (Wood et al., 1999; Avilion et al., 2003). *Sox2* has been shown to play an important role in mouse neural development – in the maintenance of the undifferentiated state of neural stem/progenitor cells, and also the neuronal differentiation of the developing eye and brain (Ferri et al., 2004; Taranova et al., 2006; Pevny and Nicolis, 2010). In all of these cases, the action of *Sox2* is dosage-dependent, with certain locations and developmental stages (postnatal hippocampus; developing eye) being especially sensitive to *Sox2* deficiency (Pevny and Nicolis, 2010). Resulting conditions include micro/anophthalmia, hippocampal abnormalities, epilepsy, and motor problems (Fantes et al., 2003; Sisodiya et al., 2006). By contrast, *Sox2* absence in other parts of the CNS show very little, or no adverse effects, which may suggest the existence of a compensation mechanism at work in those locations – something that has been suggested by Okuda et al. (2006) in work done in zebrafish (discussed below), with *Sox1* and *Sox3* as possible candidates for such compensation, also being Group B1 *Sox* genes (Miyagi et al., 2008). Normal development of the lung also appears to be dependent on Sox2 dosage – overexpression of *Sox2* has been shown to affect the branching capacity of developing lungs, leading to a severely reduced number of airways (Gontan et al., 2008). Other studies have demonstrated additional functions of *Sox2*: in dorsoventral patterning of the foregut, and the subsequent development of the

oesophagus (Que et al., 2007); and in the normal development of the trachea and lung (Que et al., 2009). *Sox2* is also expressed in the developing inner ear, with mutations leading to malformations and deafness in mice, indicating that *Sox2* is required during development of the inner ear (Hume et al., 2007; Puligilla et al., 2010), and Okubo et al. (2006) demonstrated that *Sox2* is required for the normal development of taste bud sensory cells.

Group B1 *Sox* genes are involved in the specification of certain retinal tissues in the developing eye of the chick (Ishii et al., 2009). The retina originates from a multipotential protrusion of the forebrain, and resolves into two structures, the neural retina (NR), and the retinal pigment epithelium (RPE). The study demonstrated that the B1 *Sox* family members *Sox1*, *Sox2*, and *Sox3* are downregulated in the presumptive RPE, and suggest that this mechanism is required for RPE morphogenesis and cytodifferentiation (Ishii et al., 2009). Interestingly, *Sox2* has also been shown to have the ability to reprogram differentiated RPE cells toward retinal neurons both *in vivo* and *in vitro* (Ma et al., 2009).

In *Xenopus*, *Sox1* is first expressed during embryogenesis at the early gastrula stage, and is maintained throughout development (Nitta et al., 2006). It is expressed in the anterior neural plate at stage 13, and then restricted to the brain and eye by stages 17 and 23. At stage 28 of development, it is expressed in the forebrain, hindbrain, and optic vesicle, and by stage 35 only expressed in the brain and tail. Further observations from the same study indicated that *Sox1* induced neural tissue in the ventral epidermal region, suggesting the participation of *Sox1* in neural induction, similar to *Sox1* in other vertebrate species (Nitta et al., 2006).

Sox2 is expressed from stage 11 of development to stage 38 (Mizuseki et al., 1998). It has been shown to be expressed in the CNS, brain, optic vesicle, neural retina and the lens through various stages of development (Nitta et al., 2006).

Sox3 transcripts have been detected in the unfertilised egg right the way through to stage 38 (Penzel et al., 1997). Like *Sox1* and *Sox2*, *Sox3* is expressed in the CNS, but differ from them in that at stage 28, where *Sox1* and *Sox2* can be found in the brain and the optic vesicle, *Sox3* is found in the brain and the epidermis of the optic vessel. By stage 35, *Sox3* is found in the brain and the lens, but not in the neural retina (Nitti et al., 2006). As is the case in other vertebrates, there is a degree of overlap where at least two of the Group B1 genes are expressed simultaneously in the same location, again strengthening the suggestion that these genes are functionally interchangeable (at least in certain cases).

Work done on the zebrafish demonstrates that the B1 *Sox* genes are essential for several key processes during early embryogenesis (Okuda et al., 2010). The genes involved in this study were *Sox2*, *3*, *19a*, and *19b*, where *Sox2* and *Sox3* are pan-vertebrate, and *Sox19a* and *Sox19b* are fish-specific (Okuda et al., 2006). Observations from the 2010 study showed that only the quadruple knockdown of all four genes resulted in severe developmental abnormalities. This confirms that the Group B1 genes are highly redundant, with their encoded proteins functionally interchangeable – certainly during early embryogenesis. This mirrors observations in work done on mice, and suggests that the B1 *Sox* gene products are able to compensate for each other's loss in certain mechanisms (Okuda et al., 2010).

Phenotypic analyses from the study revealed four distinct processes that are regulated by the B1 *Sox* genes: early dorsoventral patterning; gastrulation movements; neural differentiation; and neural patterning. Acting primarily as activators of numerous downstream genes – for example, developmental transcription factor genes, signalling pathway genes, and cell adhesion molecule genes – the B1 Sox proteins play a key role in coordinating cell fate specification with embryo patterning and morphogenetic processes by controlling a wide range of developmental regulators. *Sox2* has also been shown to be expressed in the taste buds of zebrafish and neuromasts of zebrafish (Germanà et al., 2009), which again shows conservation with one of the roles of *Sox2* in mice (Okubo et al., 2006). A recent study has described the requirement of *Sox2* in the maintenance and regeneration of hair cells in the inner ear of zebrafish (Millimaki et al., 2010). In the former, whilst not required in initial development of the hair cells, *Sox2* is required for their survival, and in the latter; *Sox2* is required in the transdifferentiation of support cells into hair cells, as demonstrated by the inability to regenerate in *Sox2*-depleted embryos.

A study in medaka fish describes observations from the ectopic expression of Sox3. Results from the study indicate that expression causes ectopic lens and otic vescicle formation and dysgenesis of the endogenous eye and ear (Koster et al., 2000)

Taking all of the work together, it seems that in vertebrates, the Group B1 *Sox* genes act as a suite of genes, working together in different combinations to control the expression of a range of downstream genes. They are highly implicated in early embryonic development, maintenance of neural stem cells, and in the development of neural structures. In many cases, normal function is dosage-dependent, with

overexpression having quite profound effects, for example the overexpression of *Sox2* leads to vastly reduced airways in mice (Gontan et al., 2008). The *Sox* B1 proteins are also functionally interchangeable in some cases, with one gene being able to compensate for another's loss (Okuda et al., 2010). There is still much to learn from functional studies in the Group B1 *Sox* genes.

1.3.5.ii *Sox* Group B2

The B2 *Sox* genes are a group of transcriptional repressors that act against the Group B1 *Sox* genes in the development of the nervous system (Uchikawa et al., 1999; Sandberg et al., 2005). Homologues of both B2 genes have been found in chick and mouse (Rex et al., 1997; Hargrave et al., 2000), and *Sox21* has been found in fish (De Martino et al., 1999). These studies also found that *Sox21* is expressed broadly throughout the CNS in chick, mouse and zebrafish, particularly in the midbrain-hindbrain barrier, while *Sox14* expression is largely limited to limited populations of neurons in the developing brain and spinal cord in the chick and mouse nervous system (Hargrave et al., 2000). Expression analysis in *Xenopus* has revealed that Sox14 expression is restricted to the hypothalamus, dorsal thalamus and the optic tectum (Cunningham et al., 2008). A recent sequence analysis study has revealed remarkable evolutionary conservation between *Sox14* and its orthologues in other vertebrates (Popovic & Stevanovic, 2009).

Our understanding of the functions of the B2 *Sox* genes is not as well understood as their Group B1 counterparts, but some data is available. Chick *Sox21* has been shown

to specifically act against B1 genes, and thus promoting the onset and progression of neurogenesis in the developing CNS (Sandberg et al., 2005). In zebrafish, studies involving ectopic expression of *Sox21* suggest that it acts as a repressor in dorso-ventral patterning (Argenton et al., 2004). Sox21 is also suggested to have a role in the specification of chick inner ear sensory cells (Freeman et al., 2009). Knockdown studies involving *Sox14* have revealed disruption of hypothalamic patterning in zebrafish (Kurrasch et al., 2007).

Interestingly, however, observations from a very recent study suggest a function of *Sox21* that contradicts previous hypotheses. Rather than suppressing the activity of B1 proteins (as is the case in chicks), and therefore maintaining progenitor cells in an undifferentiated state, *Xenopus laevis Sox21* (*XlSox21*) appears to enhance the expression of SoxB1 proteins, and represses neurogenesis by preventing progenitors from further progression and differentiation (Whittington et al., 2010). Further work in the form of gain or loss of function analyses have been have been planned to examine further the role of *XlSox21*.

1.3.5.iii *Sox* Group C

The Group C *Sox* genes are co-expressed in embryonic neuronal progenitors and in mesenchymal cells in many organs (Penzo-Méndez, 2010). Expression is widespread, and with a high degree of overlap between the expression profiles of the group members (*Sox4, Sox11, Sox12*) (Dy et al., 2008).

Expression is strongest in post-mitotic neuron progenitors throughout the neural tube, in dorsal root ganglia, thalamus, retina, and cerebral and cerebellar cortex. Co-expression can be found in several tissues within developing organs, such as lung, gut, and pancreas epithelium and mesenchyme (Penzo-Méndez, 2010). Cases of non-overlapping of *SoxC* genes have also been reported. For example, *Sox11* and *Sox12*is expressed in developing palatal shelves but, with no sign of *Sox4*; and *Sox4* and *Sox12* are expressed in developing thymus, but not *Sox11*; and finally *Sox4* alone is expressed in hypertrophic chondrocytes (Reppe et al., 2000), teeth bud mesenchyme, and hair follicles (Dy et al., 2008). Expression of the *SoxC* genes becomes more restricted after birth, with high levels of *Sox4* and *Sox11* found in pancreatic islet cells (Lioubinski et al., 2003), and *Sox4* found in adult gonads and thymus (Penzo-Méndez, 2010). However, neither is found in any other adult human or mouse tissue (van der Wetering, 1993; Jay et al., 1995). Conversely, *Sox12* has been found to be expressed (albeit at a low level) in most adult human tissues (Jay et al., 1997). Expression studies suggest that *SoxC* functions are conserved through evolution across the vertebrates (Penzo-Méndez, 2010), while the combined expression patterns of the two zebrafish *Sox11* genes is similar to *Sox11* expression in mouse and chick embryos, despite having distinct expression patterns from each other (de Martino, 2000).

Some functional analyses of the *SoxC* genes have been done. Schilham et al. (1996) reported that *Sox4*-null mice die at day 14 of embryonic development, with severe deformities of the heart outflow tract, leading to circulatory failure. Also, endocardial cushions are underdeveloped, which leads to incomplete separation of the ventricles, and partial or total fusion of the aortic and pulmonary vessels. The semilunar valves are also hypoplastic, which causes arterial blood backflow. *Sox11*-null mice die

immediately after birth from similar, though less severe heart malformations (Sock et al., 2004), but also display multiple other malformations including microphthalmia, open eyelids, cleft palate, cleft lips, hypoplastic lungs, asplenia, omphalocele, undermineralised skull, and split vertebrae (Wurm et al., 2008). Work done on *Sox12* showed that no obvious malformations are associated with *Sox12*-null mice, with subjects enduring normal lifespans and fertility (Hoser et al., 2008).

Sox4 and *Sox11* appear to have major role in a number of developmental processes, however when looking at the CNS and other organs that highly expressed the three *SoxC* genes, none of the single knockout studies revealed any obvious developmental defects. This leads to speculation that these genes are functionally redundant (Penzo-Méndez, 2010). RNAi knockdown studies involving *Sox4* and *Sox11* in chick development blocked neuronal gene expression, while forced expression of *Sox4*, *Sox11* or *Sox12* resulted in neuronal gene upregulation (Bergsland et al., 2006; Hoser et al., 2008). However, some studies have been undertaken that have indicated specific roles, at least for *Sox4*. Potzner et al. (2007) suggests that *SoxC* genes may both promote differentiation of progenitor cells into neurons, and prevent differentiation into glia, based on work done on *Sox4*. Similarly, an earlier study indicated that *Sox4* alone is required for B lymphocyte differentiation, based on work with *Sox4*-null cell grafts into wild-type mice (Schilham et al., 1996). Other groups have suggested that in some specific cell lineages, *SoxC* genes are required for promoting cell proliferation or survival. For example, *Sox4* knockdown results in impaired proliferation and differentiation of osteoblasts in vitro (Nissen-Meyer et al., 2007); harvested *Sox4*-null pancreatic tissue displayed severely reduced numbers of insulin-producing β-cells

(Wilson et al., 2005); and *Sox4b* knockdown in zebrafish embryos results in loss of glucagon-producing cells (Mavropoulos et al., 2005).

Although our understanding of the functions and mechanisms therein of the *SoxC* genes is limited, we now have data that suggests the roles of these genes as regulators of cell fate, proliferation and survival in major physiological and pathological processes. It is expected that further gain and loss of function, amongst other, studies will advance our knowledge and understanding of this group of genes.

1.3.5.iv *Sox* Group D

Sox Group D is made up of three genes; *Sox5*, *Sox6*, and *Sox13*. Their expression is both overlapping and autonomous, depending on the site, and these genes have been shown to have roles in both transcriptional activation and repression in various contexts.

Individually, *Sox5* is expressed in melanoblasts; *Sox6* in erythroid cells and skeletal myoblasts; and *Sox13* is expressed in arterial walls, kidney and liver. *Sox5* and *Sox13* are co-expressed in pancreatic epithelial cells, and *Sox5* and *Sox6* are expressed together in spermatids, neurons, oligodentrocytes, and chondrocytes (reviewed in Lefebvre, 2010). Despite knowing where *Sox5*, *Sox6*, and *Sox13* are expressed, the mechanisms that govern these expression patterns are virtually unknown. Although it is thought that the *SoxD* proteins interact with the *SoxE* proteins, how these interactions come about are also still a mystery (Lefebvre, 2010).

Some of the biological functions of the *SoxD* genes have been uncovered through gene inactivation work in the mouse. Smits et al. (2001) was first, reporting on the role of *Sox5* and *Sox6* in chondrogenesis. When both genes are inactivated, death occurs three days before birth, with circulatory failure cited as an apparent cause. Although the embryos have chondrocytes, they fail to differentiate and proliferate, leading to impaired skeletal growth and ossification. Where the role of *Sox5* and *Sox6* in chondrocytes is to enhance the activation of chondrocyte markers by *Sox9*, they have a very different role in oligodendrocytes, where they repress specification (Stolt et al., 2006).

Each of the *SoxD* genes also have individual functions, as is suggested by their expression patterns. *Sox5* has been shown to directly inhibit *Sox10* activity by binding to the regulatory regions of *Sox10* target genes (Stolt et al., 2006). It is also responsible for correct development of specific neuronal cell types by controlling when critical cell fate and differentiation decisions are made (Kwan et al., 2008). Overexpression studies have also suggested that *Sox5* may also play a role in the generation of the neural crest in chick embryos (Perez-Alcala et al., 2004). *Sox6* has a key role in the production of red blood cells, directly contributing to repress embryonic globin genes, and thus allowing erythrocyte maturation (Yi et al., 2006; Dumitriu et al., 2006). *Sox6*-null mice develop cardiac conduction problems and this is likely to be responsible for their inability to thrive, and ultimately death in the second or third week of life (Hagiwara et al., 2000). Hagiwara et al. (2005) has since reported that *Sox6* facilitates cardiac and skeletal muscle differentiation in late

foetuses. *Sox13*-null mice appear normal at birth, but severe growth abnormalities rapidly develop, though the reasons for this is still unknown (Melichar et al., 2009).

Our knowledge and understanding of the SoxD proteins is still in its infancy, but there is enough data to surmise that they are biologically very important, using as yet unknown mechanisms to both enhance and repress transcription in a variety of roles. They are involved in a wide range of processes in various cell lineages, encompassing cell proliferation, survival, differentiation, and maturation.

1.3.5.v *Sox* Group E

Sox8, *Sox9* and *Sox10* make up the complement of Group E *Sox* genes. This group of genes initially attracted a lot of attention because of their roles in vertebrate testis formation, but their importance in other processes such as neural crest formation and nervous system development have more recently come about.

Beginning with testis formation, the importance of *Sox9* in this process became clear very early on, whilst the role of *Sox8* in normal testis development and fertility has only recently become apparent (Barrioneuvo & Scherer, 2010). So far, no role has been identified for *Sox10* in testis development (Cory et al., 2007). Ectopic expression of *Sox9* has been shown to effect testis development in XX gonads, despite the absence of *Sry* (known as the master sex determining gene in mammals) (Vidal et al., 2001). This suggests that *Sox9* is the only downstream gene of *Sry* required to initiate male development. When *Sox9* levels reach a critical threshold, it enters a positive

regulatory loop at the site of a testis-specific enhancer of *Sox9* (TES), and this maintains the expression on *Sox9* (Sekido & Lovell-Badge, 2008). During the differentiation of Sertoli cells (cells that are required for the development of spermatozoa), pre-Sertoli cells come together and form the testis chords, which eventually mature to form the seminiferous tubules (the site of spermatogenesis). *Sox9* is thought to be the key factor regulating relevant gene activity during this process (Barrionueva & Scherer, 2010), and continues to be expressed after testis chord formation, suggesting additional roles in proliferation and maturation.

Sox8 expression in Sertoli cells begins just after *Sox9* is upregulated, and has been hypothesised, even, to be directly regulated by Sox9 (Chaboissier et al., 2004). This expression continues during embryonic and postnatal gonadal development (Schepers et al., 2003). *Sox8*-null males are initially fertile, but develop progressive spermatogenic failure (O'Bryan et al, 2008). Similar to other *Sox* genes, there is a suggestion of functional redundancy between *Sox8* and *Sox9*. This is indicated by observations of more severe phenotypes where there is inactivation of both genes – incomplete knockout of *Sox9* on a *Sox8*-null background leads to an XY sex reversal phenotype with little or no testis chords; and specific inactivation of *Sox9* on a *Sox8* mutant background leads to progressive degeneration of testis chord, and infertility (Chaboissier et al., 2004; Barrionuevo et al., 2009). The control of *Amh* (anti-Müllerian hormone) is also important during sexual differentiation, as it causes the regression of the Müllerian ducts, the starting point of the female internal genitalia. Studies indicate that *Sox8* and *Sox9* work together to initiate and regulate *Amh* expression (Chaboissier et al., 2004; Barrionuevo et al., 2006; Barrionuevo et al., 2009; Schepers et al., 2003; reviewed in Barrionuevo & Scherer, 2010).

The neural crest (NC) is a group of embryonic precursor cells that are found at the crest of the closing neural tube (precursor of the spinal cord). These cells undergo what is known as an epithelial to mesenchymal transition (EMT) and migrate throughout the early embryo, differentiating into a diverse range of structures (reviewed in Haldin & LaBonne, 2010). The SoxE transcription factors are the earliest indicators that NC precursor cells are competent to give rise to the definitive neural crest (Sauka-Spengler & Bronner-Fraser, 2008). In birds and mammals, this is *Sox9*, and in *Xenopus*, *Sox8* (Hong & Saint-Jeannet, 2005). Indications are that the primary role of *Sox10* in neural crest formation is in later regulatory events (Haldin & LaBonne, 2010).

The *SoxE* genes, along with the *SoxB* genes, play important roles in both the peripheral (PNS) and the central (CNS) nervous systems. The Sox10 protein is of particular importance in PNS development in all vertebrates analysed (Stolt & Wegner, 2010). In migrating NC cells, it is key to cell survival and inhibits premature neuronal differentiation (Kim et al., 2003). Sox10 deficiency in mice leads to a complete loss of all PNS glia (Britsch et al., 2001), indicating that it is required for glial specification. Evidence in zebrafish suggests a role for Sox10 in the specification of a number of sensory neurons in the dorsal root ganglia (Carney et al., 2006). The absence of Sox10 most affects the enteric nervous system (ENS), where enteric ganglia fail to develop (Maka et al., 2005). In humans, mutations in *Sox10* contribute to the PCWH syndromes (Peripheral Demyelinating Neuropathy, Central Dysmyelinating Leukodystrophy, Waardenburg Syndrome, and Hirschsprung

Disease) (Inoue et al., 2004). *Sox10* also directly regulates the expression of several genes responsible for myelin formation in the CNS (Stolt et al., 2002).

1.3.5.vi *Sox* Group F

The *SoxF* genes (*Sox7*, *Sox17*, *Sox18*) have key roles in cardiovascular development, directing cell differentiation in the developing heart, blood vessels and lymphatic vessels (Francois et al., 2010). Disruption of *Sox17* expression in embryonic stem cells appear to block cardiac myogenesis by affecting signals that drive cardiac specification in the primitive mesoderm (Liu et al., 2007), whereas knockdown of the *Xenopus Sox7* and *Sox18* orthologues results in reduced expression of cardiac markers (Zhang et al., 2005). *Sox18*/*Sox18* double-null mice display severe defects in the formation of anterior vessels that correlate with reduced endocardial cell differentiation and atypical fusion of the endocardium (Francois et al., 2010). Mutations in *Sox18* also result in severe vascular defects of a dual phenotype, characterised by impairment of both blood and lymphatic vasculature (Francois et al., 2010). *Sox18*-null mice die *in utero* at around 14.5 dpc, and have been shown to completely lack lymphatic vasculature (Francois et al., 2008). In zebrafish, double knockdown of *Sox7* and *Sox18* is also characterised by a blood vascular phenotype. Multiple fusions between the axial vessels block blood circulation, and implies redundant roles for *Sox7* and *Sox18* in arterio-venous differentiation of endothelial cells (Pendeville et al., 2008).

1.3.5.vii Vertebrate *Sox* Group G

Sox15 is the only member of the mammalian *SoxG* group. Its orthologues are zebrafish *Sox19a*/*b*, and *Xenopus SoxD*. These orthologues are highly expressed in the CNS (Mizuseki et al., 1998; Okuda et al., 2006), whereas it appears to only be strongly expressed in the placenta in mice (Yamada et al., 2006), and in fetal testis in humans (Hiraoka et al., 1998). It is interesting to note that the *SoxB1* genes *Sox2* and *Sox3* are also expressed in the placenta (Wood & Episkopou, 1999), given that the *SoxG* group is most closely to the *SoxB1*group of genes. Studies suggest that *Xenopus SoxD* is required for anterior neural development (Mizuseki et al., 1998), but in mice, *Sox15* has been shown to be involved in skeletal muscle regeneration (Lee et al., 2004).

Sox15-null mice appear to be healthy and fertile with normal skeletal muscle development, but exhibit delayed skeletal muscle regeneration after crush injury (Maruyama et al., 2005), also indicating that *Sox15* is involved in skeletal muscle regeneration.

Although *Sox15* is strongly expressed in the placenta, and is implicated in placental development (Yamada et al., 2006), *Sox15*-null mice are still fertile as mentioned above, suggesting that the placenta develops normally despite the absence of *Sox15*. This indicates possible functional redundancy between Sox15, Sox2 and Sox3 within the organ (Ito, 2010) given that the latter two are *SoxB1* genes also expressed in the placenta. The exact functions of the *Sox* genes in the placenta are still to be elucidated,

and would require the production and analysis of double and triple knockout mice for *Sox15*, *Sox2* and *Sox3*.

1.3.5.viii *Sox* Group H

Very little is known about the sole representative of *Sox* Group H, *Sox30*. Until recently, it has been thought that *Sox30* exists only in mammals – reports suggesting its presence in zebrafish (De Martino, 1999) turned out to be a case of mis-identification, with *Sox21*. It has recently been identified in the Nile tilapia, *Oreochromis niloticus*, a discovery made quite by accident as it was done during attempts to clone *Sox9b* in the species (Han et al., 2010), and further investigations have revealed that it is in fact quite widely found throughout the animal kingdom, including some species of fish. Studies have suggested that *Sox30* may be involved in mammalian spermatogonial differentiation and spermatogenesis (Osaki et al., 1999; Ballow et al., 2006). In the Nile tilapia, *Sox30* has been detected in male gonads from about 10 days after hatching (dah), which excludes it from having a role in sex determination, as the critical period for molecular sex determination is 5 dah (Han et al., 2010). The same study has also shown that *Sox30* is also expressed in the somatic cells, and especially the steroidogenic cells of the ovary, indicating an important role for *Sox30* as an important regulator of somatic differentiation and steroidogenesis in female fish too. It has been proposed that *Sox30* may be involved in gonadal differentiation and development in different sexes, at different stages, and in different cell types of gonads in the animal kingdom (Han et al., 2010).

In summary, and taking data on all of the *Sox* groups together, it is apparent that the *Sox* proteins play key roles in specifying cell fate – driving terminal differentiation of pluripotent embryonic stem cells into many cell types. Mutations in several of these genes result in severe clinical syndromes in humans, including disorders of sexual development. Key features of this suite of genes have come to light – for example the functions of the signature HMG box, critical in DNA bending, protein-protein interaction, and protein nuclear localisation; the fact that there is close sequence homology, certainly within the HMG box, throughout evolutional history; the notion that *Sox* proteins accomplish most of their functions by working in pairs and synergising with many types of transcription factors; or that proteins within the same group often have overlapping expression patterns and functions, allowing for a degree of functional compensation or redundancy. It does appear, however, that each group has a functional 'niche', whereby specific processes or pathways are governed (in the main part, at least) by a particular group of the *Sox* family. In the context of this thesis, where interest is focused on the processes responsible for sexual development, although not exclusively limited to these, the group of genes that come to the fore are the *Sox* group E genes, which include *Sox8*, *Sox9*, *Sox10*.

1.4 Salmonid gender studies

In any study into the genetic mechanisms of a given process within a species, it is important to at least quantify, if it is not possible to compare, the differences and similarities between the organism of interest and its related species. In the current study, a key aim is to elucidate a sex-linked marker that can be used to determine the sex of an Atlantic salmon individual, merely by its presence. It is logical then, to explore any work that has been done on related species, and also to determine whether any significant findings from these studies show any correlation with the situation in Atlantic salmon. This chapter summarises some of the relevant findings from genetic studies on salmonid species.

As previously discussed, the closest species to Atlantic salmon, *Salmo salar*, are those belonging to the *Oncorhynchus* and *Salvelinus* genera. The Pacific salmon (genus *Oncorhynchus*) form the largest group of salmonids; species include:

Oncorhynchus tshawytscha – Chinook salmon

Oncorhynchus mykiss – Rainbow trout (also called Steelhead or Ocean trout)

Oncorhynchus keta – Chum salmon

Oncorhynchus clarki – Cutthroat trout

Oncorhynchus masou – Masu salmon (also Cherry salmon)

Oncorhynchus gilae – Gila trout

Oncorhynchus nerka – Sockeye salmon

Oncorhynchus kisutch – Coho salmon

Oncorhynchus gorbuscha – Pink salmon

Oncorhynchus chrysogaster – Mexican golden trout

All of the Pacific salmon species have been successful in their native river systems, and are of comparative prevalence. As such, genetic studies within the *Oncorhynchus* genus have not been centred upon any particular species, although it appears that slightly more literature is available relating to Rainbow trout and Chinook salmon.

The Charrs (genus *Salvelinus*) form the next largest group of salmonids; species include:

Salvelinus alpinus – Arctic charr

Salvelinus namaycush – Lake trout

Salvelinus fontinalis – Brook trout

Salvelinus malma – Dolly Varden trout

Salvelinus confluentus – Bull trout

Salvelinus japonicus – Kirikuchi charr

Salvelinus leucomaenis – Whitespotted charr

Much of the genetic research concerning these species has been concentrated on the Arctic charr, Lake trout and Brook trout, as they are the most widely distributed and most prevalent species in the genus. They are also important food fish in their native range.

Far more literature can be found detailing work done on the numerous *Oncorhynchus* and *Salvelinus* species than on the comparatively few *Salmo* species, and here follows

a summary of some of the key findings from such studies, especially those of particular interest to the current study.

We have already established that a wide range of modes of sex determination exist in fish. These include purely environmental mechanisms; primarily genetic sex determination (but modulated by environmental factors); and rigid genetic sex determination.

Within genetic sex determination, there may be an even greater variety of mechanisms governing gender. These include polygenic inheritance, male *or* female heterogamety, multiple sex chromosomes, and autosomal factors. These are reviewed in (Devlin & Nagahama, 2002). Differing mechanisms may occur in closely related species such as the various Tilapia (*Oreochromis*) species. Different mechanisms have even been observed among populations of the same species (for example the Platyfish, *Xiphophorus maculatus*). Furthermore, not many species have evolved morphologically distinguishable sex chromosomes (Beçak, 1983) – this is in stark contrast to higher vertebrates, where differences in the sex chromosomes are generally far more apparent.

Currently, the most widely accepted hypothesis for Salmonids is that they operate an (XX:XY) male heterogamety system, although differentiated sex chromosomes have still not been identified in most species (Devlin & Nagahama, 2002; Davidson et al., 2008). Most of the evidence for male heterogamety comes from analysis of sex ratios in the progeny of hormonally sex-reversed individuals. For example, sex-reversed females of Rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus*

tshawytscha), Coho salmon (*Oncorhynchus kisutch*) and Atlantic salmon (*Salmo salar*) produce all-female progeny when crossed with normal (genotypic and phenotypic) females. This indicates that females are homogametic XX (Hunter et al., 1982, 1983; Johnstone and Youngson, 1984; Johnstone et al., 1987; Devlin et al., 2001).

Heteromorphic sex chromosomes are believed to have evolved from a homomorphic chromosome pair as a consequence of reduced recombination. A hypothesis is that sexually antagonistic genes located on the sex chromosomes promoted the evolution of mechanisms that suppress crossover (Rice, 1987). Chromosomal rearrangements and heterochromatic additions (which are known to alter recombination) are suggested to serve a primary role in the differentiation of heteromorphic chromosomes (Reed et al., 1995). In meiotic studies of the deer mouse (*Peromyscus beatae*) (Sudman & Greenbaum, 1990), there were found to be modifications in sex chromosome pairing resulting from varying amounts of heterochromatin. In a more relevant context, Phillips and Ihssen (1985), working on Lake trout (*Salvelinus namaycush*) hypothesised that the accumulation of heterochromatin on the X chromosome had operated to reduce crossing over between the homologues, and thus facilitating differentiation.

As previously mentioned, morphologically distinct sex chromosomes have been detected in only a small number of species. The sex chromosomes of Lake trout (*Salvelinus namaycush*) and Brook trout (*Salvelinus fontinalis*), which are closely related, were identified by an X-specific heterochromatin block at the end of the short arms of the largest pair of submetacentric chromosomes present in both species

(Phillips et al., 2002). There are reports of differences in diploid chromosome number between males (57) and females (58) in Sockeye salmon (*Oncorhynchus nerka*), which is thought to be resultant from Y-autosome fusion (Fukuoka, 1972; Thorgaard, 1978). In Rainbow trout (*Oncorhynchus mykiss*) differences in size have been observed in the short arm of a small subtelocentric pair of homologous chromosomes between the sexes (Thorgaard, 1977). It is of interest to note that, despite these indications of an apparent migration towards male heteromorphism, there have been observed cases where this heteromorphic condition has been lacking in individuals – namely, in Rainbow trout (Thorgaard et al., 1983) and Sockeye salmon (Fukuoka, 1972). This would indicate that chromosome rearrangements involved in differentiating the sex chromosomes are still in the process of fixation. Furthermore, a comparative genome analysis of the primary sex-determining locus in salmonid fish (Woram et al., 2003) has determined that different Y-chromosomes have evolved in different species of Salmonids.

Sex-specific or sex-linked repetitive DNAs have been identified in a number of species – for example; Medaka, *Oryzias latipes* (Matsuda et al., 1998); Guppy, *Poecilia reticulata* (Nanda et al., 1992); and more interestingly due to being a salmonid, the Chinook salmon, *Oncorhynchus tshawytscha* (Stein et al., 2001; Du et al., 1993). In fact, there are two sequences that are of interest in Chinook salmon: *OtY1*, a sequence that is part of an 8-kilobase repeat – *OtY8* – on the Y chromosome (Stein et al., 2001), and *GH-ΨY* (Du et al., 1993), a growth-hormone pseudogene sequence – both of which are closely linked to the sex-determination locus on the Y chromosome of Chinook salmon. The *OtY1* sequence was first characterised by

Devlin et al. (1998), and *GH-ΨY* by Du et al. (1993). In both cases, initial observations indicated that the sequences are closely linked to the Y-chromosome.

Stein et al. (2001) used the fluorescence in situ hybridisation technique to identify the Y chromosome in Chinook salmon. They used the *OtY1* sequence described above as the male-specific probe, and on analysis of the results concluded that the sequence is specific to the Y chromosome, confirming the earlier work done by Devlin et al. (1998). At the same time, follow-up work by Devlin et al. (2001) based on previous findings (1998) reported that *GH-ΨY* is closely linked to the sex determination locus on the Y chromosome, and also to *OtY1*. This data suggests that the *OtY1* and *GH-ΨY* sequences could be used as male-specific markers in Chinook salmon.

In terms of homology with other Salmonid species, indications are that *Salmo* and *Salvelinus* species do not possess the *GH-ΨY* sequence. Not only that, but *GH-ΨY* is also not present in all *Oncorhynchus* species (Devlin et al., 2001). In the case of *OtY1*, it would appear that similar sequences have been even more difficult to identify – sequences related to *OtY8* (of which *OtY1* is a part) are present on the autosomes or X-chromosome in many of the Salmonid species, but extensive amplification has not been observed outside of the Y-chromosome of Chinook salmon (Devlin et al., 1998).

More recent studies on *OtY1* and *GH-ΨY* have found that rather than being robust sex-linked genetic markers, there is a lack of the reported specificity in other populations of Chinook salmon (Chowen & Nagler, 2005). These results were echoed by another study, which showed that Y-chromosome linkage of the two sequences vary considerably between different populations of the species (Devlin et al., 2005). In fact,

these observations appear to complement the findings of work done on Rainbow trout (Phillips, 2001), where Y-chromosome variation between different populations is reported. This extensive variation in the Y-chromosome and its associated sequences between populations of the same species has been suggested to potentially lead to speciation in the future.

Taking current cytogenetic data into account, it would appear that salmonid species are currently at the early stages of sex chromosome differentiation. This is further supported by the viability and fertility of YY males (Chevassus et al., 1998), which suggests that X- and Y-chromosomes still share a similar inventory of functional genes (Woram et al., 2003).

Genetic linkage maps have been constructed for a number of species, including rainbow trout (Sakamoto et al, 2000), brown trout (Gharbi, 2006), Atlantic salmon (Moen et al., 2004) and Arctic Charr (Woram et al., 2004). Taken together, these data indicate a lack of conservation in the sex determining locus (*SEX*) in salmonids. An early study showed that sex-linked allozyme markers in Arctic charr were not linked to *SEX* in lake trout and brook trout (May et al., 1989). Other studies reported similar observations – the growth hormone marker *GH-ΨY* (discussed above), believed to be sex-linked in coho salmon, Chinook salmon and masu salmon were not found to be so in amago salmon and rainbow trout (Forbes et al., 1994; Nakayama et al., 1999; Zhang et al., 2001). Additionally, a minisatellite locus shown to be tightly linked with *SEX* in brown trout was mapped to an autosomal pair in Atlantic salmon (Prodöhl et al., 1994; Taggart et al., 1995). Despite these indications that *SEX* is generally not conserved in salmonids (at least in these species), it appears to be so in rainbow trout

and Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*) (Alfaqih et al., 2008). Woram et al. (2003) compared the mapping of microsatellite markers linked to *SEX* in several salmonid species (*Salvelinus alpinus*, *Salmo trutta*, *Salmo salar*, *Oncorhynchus mykiss*), and found that conservation of synteny of *SEX* and microsatellite markers was lacking among the species examined. Additionally, *SEX*-linked microsatellite markers able to amplify across salmonids map to autosomal, homologous linkage groups in other species. This data further supports the hypothesis that these closely related species of salmonids have evolved different sex chromosomes.

SEX in the Atlantic salmon has been mapped to Atlantic salmon linkage group 1 (ASL1), and is associated with several microsatellite markers (Artieri et al., 2006). The same study has identified chromosome 2 as the sex chromosome in Atlantic salmon, and have proposed a physical location of *SEX* as being on the long arm of chromosome 2, between the Ssa202DU microsatellite marker and the large region of DAPI stained heterochromatin.

Despite all of the work in elucidating the nature and location of the sex determining locus in salmonids, and more specifically, in Atlantic salmon, there are still many questions to be asked (and answered) in relation to the mechanisms that govern sex determination itself, and indeed, the master switch (or switches) that turn these mechanisms on.

1.5 Suppressive Subtractive Hybridisation (SSH)

Suppressive subtractive hybridisation (SSH) is a powerful process that enables the comparison of two populations of mRNA, and obtaining clones of sequences that are expressed in one population but not the other. This feature is potentially very useful for the current study; as it holds promise to elucidate sequences that are expressed in the male population of the Atlantic salmon species but not in the female. The resultant sequences could therefore potentially be strong candidates as sex-linked genetic markers for the species.

The basic theory behind the technique is actually quite simple, and can be divided into three main components. First, the mRNA sequences must be converted into cDNA. Next, the two cDNAs are hybridised; and then lastly the hybrid sequences are removed. This leaves the unhybridised cDNAs, representing sequences and genes expressed in one but absent from the other population of mRNA.

Traditional subtractive hybridisation methods are not well suited for the identification of rare messages, and usually require several rounds of hybridisation. The technique used in this study uses a unique method where differentially expressed sequences are selectively amplified, overcoming the technical limitations of traditional subtraction methods (Diatchenko et al., 1996; Gurskaya et al., 1996). The suppression PCR technique (discussed later) prevents amplification of undesirable elements during the enrichment of target molecules.

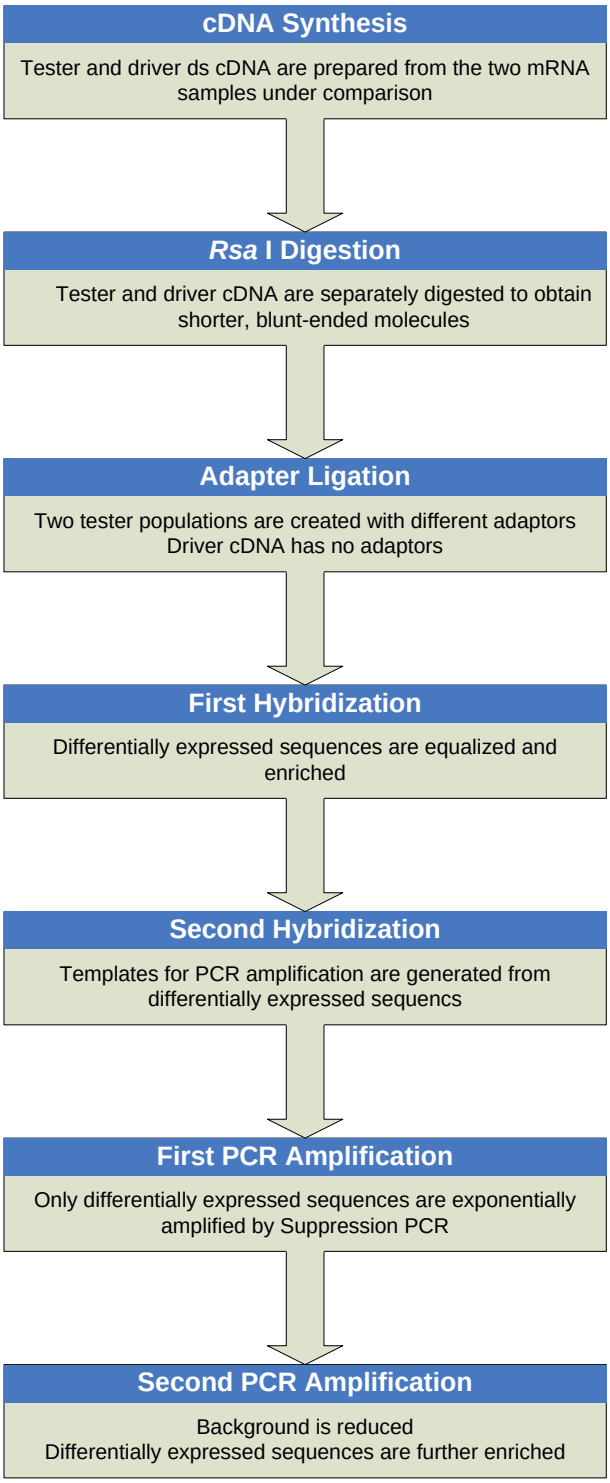


Fig. 1.5.1

Overview of Suppressive Subtractive Hybridisation Method

The first step in SSH involves making high quality cDNA from ~2μg of Poly A^+ RNA extracted from the two tissues being compared (in this case, tissue from a male Atlantic salmon, and tissue from a female Atlantic salmon). When synthesised, the cDNA containing the 'projected' differentially expressed transcripts is referred to as the tester, and the reference cDNA as the driver. Primarily for the current study, the male component has been designated the tester; the female component the driver, though these designations are also reversed to uncover any differentially expressed transcripts in the female cDNA. Therefore, both male and female sequences are used in the experiment as both tester and driver.

The tester and driver cDNAs are digested with *Rsa* I, a four-base-cutting restriction enzyme that yields blunt-ended fragments. The purpose of this is simply to cut the cDNA into smaller fragments, for more effective amplification during PCR.

The following parts of this method are duplicated – using the female cDNA as the tester and the male cDNA as the driver – and run alongside the outlined experiment (see Fig. 1.5.2), ultimately to yield differentially expressed sequences from both the male and female cDNA.

The two *Rsa* I-digested tester cDNAs are then sub-divided into two aliquots and each is ligated with a different cDNA adaptor; Adaptor 1 and Adaptor 2R. With no phosphate groups on the ends of the adaptors, only one strand of each adaptor attaches to the 5' ends of the cDNA. The two adaptors have stretches of identical sequences, which allow annealing of the PCR primer once the recessed ends have been filled in.

In the first hybridisation, an excess of driver is added to each sample of tester, heat denatured, then allowed to anneal, generating four types of molecule in each sample:

- Type **a** – Unpaired tester cDNA sequences with Adaptor 1 or Adapter 2R.
- Type **b** – Paired tester cDNA sequences with Adaptor1 or Adaptor 2R.
- Type **c** – Tester cDNA sequences with Adaptor 1 or Adaptor 2R, paired with Driver cDNA.
- Type **d** – Paired or unpaired Driver cDNA sequences.

The concentration of high- and low-abundance sequences are equalized among the Type **a** molecules because reannealing is faster for the more abundant molecules due to the *second-order kinetics of hybridisation* (James & Higgins, 1985). At the same time, Type **a** molecules are significantly enriched for differentially expressed sequences while cDNAs that are not differentially expressed form type **c** molecules with the driver.

Following this, in the second hybridisation, the two primary hybridisation samples and are mixed together *without* denaturing. Here, only the remaining equalized and subtracted single-stranded (ss) tester cDNAs can reassociate and form new hybrids (Type **e**). These new hybrids are double-stranded (ds) tester molecules with different ends that correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA is added – again, without denaturing the subtraction mix, to further enrich the Type **e** component for differentially expressed sequences. After the ends are filled in by DNA polymerase, the differentially expressed sequences (Type **e**) have different annealing sites for the nested primers on their 5’ and 3’ ends.

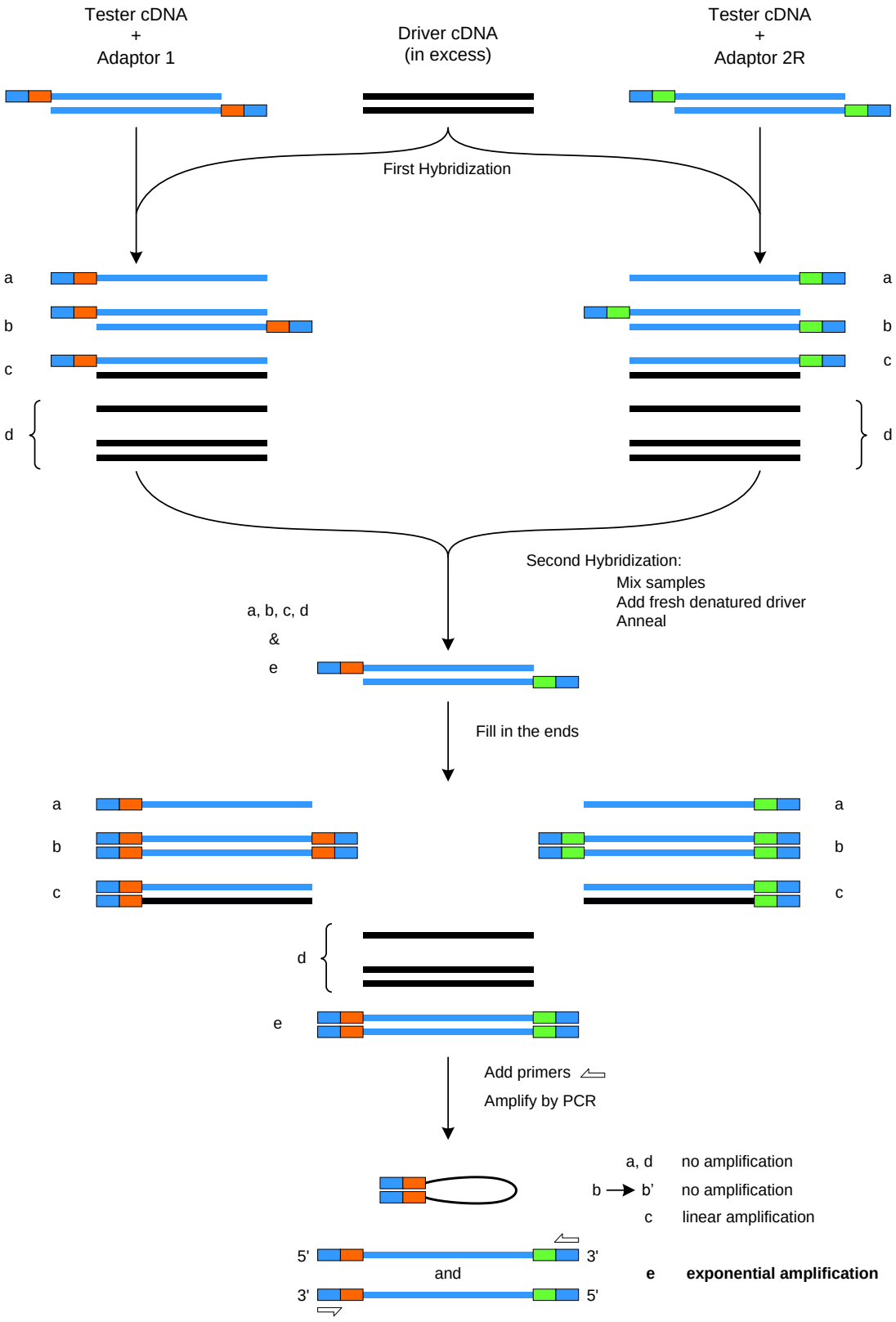
The entire population of molecules (Types **a**, **b**, **c**, **d**, **e**) then undergoes a PCR reaction to amplify the desired differentially expressed sequences. During this PCR, Type **a** and Type **d** molecules cannot be amplified, as they are missing primer annealing sites. Due to the suppression PCR process (see later), most Type **b** molecules form a pan-like structure that prevents their exponential amplification. Type **c** molecules have only one primer annealing site and so only amplify linearly. It is only the Type e molecules – the equalized differentially expressed sequences with two different adaptors – that amplify exponentially.

A secondary PCR amplification is the next step, using nested primers to further reduce any background PCR products and enrich (or select) for differentially expressed sequences.

In theory, at the end of the process, there are two populations of differentially expressed sequences specific to either the male or female part of the Atlantic salmon genome – male, if the products come from the use of male cDNA as the tester; female if the products come from the use of female cDNA as the tester.

Fig. 1.5.2 (overleaf)

Schematic diagram of Suppressive Subtractive Hybridisation (duplicated in a parallel experiment using tester as driver, and driver as tester)



Schematic Diagram of Suppression Subtractive Hybridization

Type **e** molecules are formed only if the sequence is upregulated in the tester cDNA. Solid lines (blue or black) represent the *Rsa* I-digested tester or driver cDNA. Blue boxes represent the outer part of the adaptor 1 and 2R longer strands and corresponding PCR primer 1 sequence. Orange boxes represent the inner part of the Adaptor 1 and the corresponding Nested PCR primer 1 sequence. Green boxes represent the inner part of Adaptor 2 and the corresponding Nested PCR primer 2R sequence

The adaptors used in the process are engineered to prevent undesired amplification of common transcripts during PCR by a method called suppression PCR. This apparent suppression transpires when complementary sequences are present on both ends of a single-stranded cDNA. The properties of the adaptors are such that during every annealing step of the PCR cycle, the hybridisation kinetics strongly favour the formation of a pan-like secondary structure (over the annealing of primers) which prevents the amplification of that particular cDNA sequence. On the occasion where a primer does manage to anneal and extend, the newly synthesized strand will also have the complementary sequences on either end and thus face the same negative kinetic favourability that leads to the suppression-effecting, pan-like structure. Non-specific amplification is therefore efficiently suppressed during PCR, and the specific amplification of cDNA molecules with different adaptors at either end can proceed normally.

The 5’ ends of Adaptor 1 and Adaptor 2R are an identical stretch of 22 nucleotides, which means that primary PCR only requires one primer for amplification. This eliminates the problem of primer dimerization (Lukyanov et al., 1995). Additionally, a slight suppression PCR effect is introduced by the identical sequences on the 3’ and 5’ ends of the differentially expressed molecules. These sequences are the same length as PCR primer 1, and so the suppression effect is only significant for very short cDNAs (under 200 nucleotides). This is because the formation of pan structures is more efficient for shorter sequences (Lukyanov et al., 1995). As a result, the longer molecules are preferentially enriched, overcoming the inherent tendency of the subtraction procedure to favour short cDNA fragments. These shorter fragments are more efficiently hybridised, amplified, and cloned than longer fragments.

1.6 Statement of Aims

From the foregoing introduction it will be clear that salmon gender is both poorly understood, and likely to be complex. As a first step towards a better understanding of salmon gender, the aim of this thesis is to develop a non-destructive method (i.e. not resulting in the animal's death) for preparing nucleic acid from fish. This DNA (or RNA) will then be assayed by various molecular techniques with the aim of identifying markers specific for gender. Achieving these aims would represent partial success in this project, whilst complete success would mean we understood how gender was determined in salmon.

2. Materials and Methods

Described in this chapter are the materials and methods used for the extraction and purification of genomic DNA, total RNA, and PolyA$^+$ RNA from Atlantic salmon tissue, and for agarose gel electrophoresis of the products of PCR reactions. Also included are the materials and methods involved in each specific strand of experimental work undertaken in the present study.

2.1 Tissue Digestion for DNA Extraction and Purification

Materials

Water bath set at 37°C (Progen); Atlantic salmon tissue (various sources); 1.5ml Microcentrifuge tubes (Fisher Scientific); Fisherbrand Pipettors and filter tips (Fisher); Nitrile latex-free gloves (Fisher); 0.2% DTT (Sigma-Aldrich); 0.5M EDTA (Fisher); 1 mg/ml Proteinase K (Sigma-Aldrich); 1M Sodium Chloride (Sigma-Aldrich); 10% SDS (Fisher); 1M Tris buffer (Fisher).

Method

A 500µl digestion solution was made up in a 1.5ml microcentrifuge tube, containing 50mM Tris (pH8); 50mM EDTA (pH8); 200mM NaCl; 1% SDS; 0.2% DTT; 1mg/ml Proteinase K. This was then mixed thoroughly by vortexing, and a 0.5cm^3 piece of Atlantic salmon tissue was added to the digestion solution. This was then incubated in a covered water bath overnight at 37˚C.

2.2 DNA Extraction

Materials

Microcentrifuge (Progen); 1.5ml Microcentrifuge tubes (Fisher); Fisherbrand Pipettors and filter tips (Fisher); Nitrile latex-free gloves (Fisher); digested Atlantic salmon tissue (Various sources); 8M ammonium acetate (Fisher); Chloroform:Isoamylalcohol (Sigma-Aldrich); Phenol:Chloroform:Isoamylalcohol (Sigma-Aldrich); 99.9% Ethanol (Fisher); Tris-EDTA (TE) buffer (Fisher).

Method

The lysate was centrifuged at 13,200rpm for 30 min to pellet the undigested material, and the supernatant was transferred into a new microcentrifuge tube. An equal volume of Phenol:Chloroform:Isoamylalcohol was added and the mixture was shaken vigorously by hand for 10 min, then centrifuged for 10 min at 13,200rpm. The top layer was transferred into a new tube, and the bottom layer was discarded. An equal volume of Chloroform:Isoamylalcohol was added to the supernatant and the tube was shaken vigorously by hand for 10 min, then centrifuged for a further 10 min at 13,200rpm. The top layer was transferred into a new tube and the bottom layer was discarded. A quarter volume of 8M ammonium acetate was added and the solution was cooled on ice for 30 min, before being centrifuged for 10 min at 13,200rpm. The supernatant was transferred into a new tube and the pellet was discarded. A 2x volume of >95% ethanol was added to the supernatant, and left on ice for 1hr. The mixture was then centrifuged at 13,200rpm for 20 min. The supernatant was removed and discarded, the pellet was washed with 500µl of 70% ethanol, and then centrifuged at

13,200rpm for 10 min. The ethanol was removed and the pellet allowed to dry at room temperature. The pellet was then resuspended in 100µl TE buffer and stored at -20°C.

2.3 RNA Purification

Materials

Polypropylene Pellet Pestle (Sigma-Aldrich); 1.5ml Microcentrifuge tubes (Progen); Desktop microcentrifuge (Progen); Pipettors and Filter-tips (Fisher); Nitrile powder-free gloves (Fisher); *Spin Cartridges (Invitrogen); Chloroform (Sigma-Aldrich); >95% ethanol (Fisher); *TRIzol Reagent (Invitrogen); *Wash Buffer #1 (Invitrogen); *Wash Buffer #2 (Invitrogen); RNase-free water (Sigma-Aldrich).

*Supplied as part of the *TRIzol Plus RNA Purification Kit*, Invitrogen.

Method

100mg of Atlantic salmon tissue was homogenised in 1 ml TRIzol reagent, using a pellet pestle. The lysate was then incubated at room temperature for 5 min, then 200µl chloroform was added and the tube was shaken vigorously by hand for 30 sec. The mixture was incubated at room temperature for 3 min, then centrifuged at 12,000g for 15 min at 4°C. The colourless upper phase was transferred to a fresh tube, an equal volume of 70% ethanol was added, then the mixture was mixed thoroughly by vortexing.

700µl of the sample was transferred to an RNA Spin Cartridge, then centrifuged at 12,000*g* for 15 sec at room temperature. The flow-through was discarded.
Any remaining sample was then transferred to the RNA Spin Cartridge and centrifuged at 12,000*g* for 15 sec at room temperature. The flow-through was discarded.

700µl of Wash Buffer I was added to the RNA Spin Cartridge, then centrifuged at 12,000*g* for 15 sec at room temperature. The collection tube was discarded. The RNA Spin Cartridge was then placed into a clean RNA Wash Tube, and 500µl of Wash buffer II was added to the RNA Spin Cartridge. It was then centrifuged at 12,000*g* for 15 sec at room temperature, and the flow-through was discarded. Another 500µl of Wash buffer II was added to the RNA Spin Cartridge and centrifuged at 12,000*g* for 15 sec at room temperature. The Wash Tube was discarded, and the RNA Spin Cartridge was then placed into a clean RNA Recovery Tube.

30µl of RNase-free water was added to the centre of the RNA Spin Cartridge membrane and incubated for 1 min. It was then centrifuged for 2 min at 12, 500*g* at room temperature and the RNA Spin Cartridge was discarded, leaving purified total RNA contained in the RNA recovery tube. The sample was then stored at -70°C.

2.4 Isolation of PolyA$^+$ mRNA from total RNA

Materials

37°C water bath (Grant); air incubator set at 70°C (Memmert); vortex (Fisherbrand); microcentrifuge (Progen); pipettor and filter-tips (Fisher); *Spin Columns (Qiagen); Atlantic salmon total RNA (self-extracted); *Oligotex suspension (comprising 10% w/v suspension of Oligotex particles in 10mM Tris-Cl, pH 7.5; 500nN NaCl; 1mM EDTA; 0.1% SDS; 0.1% NaN_3) (Qiagen); *Buffer OBB (comprising 20mM Tris-Cl, pH 7.5; 1M NaCl; 2mM EDTA; 0.2% SDS) (Qiagen); *Buffer OEB, containing 5mM Tris-Cl, pH 7.5 (Qiagen); *Buffer OW2 (comprising 10mM Tris-Cl, pH 7.5; 150mM NaCl; 1 mM EDTA) (Qiagen); RNase-free water (Sigma).

*Supplied as part of the *Oligotex mRNA Purification Kit*, Qiagen

Method

The Oligotex Suspension was heated to 37°C in a water bath, then mixed by vortexing and placed at room temperature. Buffer OEB was heated to 70°C in a water bath. Buffer OBB was redissolved by warming to 37°C in a water bath, to disperse precipitate formed in storage.

1mg of Atlantic salmon total RNA was pipetted into an RNase-free 1.5ml microcentrifuge tube, and the volume was made up to 250µl with RNase-free water. 250µl of Buffer OBB and 15µl of Oligotex Suspension was added, and the solution was mixed thoroughly by pipetting. The sample was then incubated at 70°C in a water bath for 3 min. The sample was removed from the water bath and placed at room temperature for 10 min to allow hybridisation between oligo dT_{30} of the Oligotex

particle and the poly-A tail of the mRNA. The Oligotex-mRNA complex was then pelleted by centrifugation for 2 min at maximum speed, and the supernatant was removed. The pellet was then resuspended in 400µl of Buffer OW2 by pipetting, and transferred onto a small spin column placed in a 1.5ml microcentrifuge tube. This was centrifuged for 1 min at maximum speed. The spin column was transferred to a new RNase-free 1.5ml microcentrifuge tube, 400µl of Buffer OW2 was added to the column, and then centrifuged for 1 min at maximum speed. The flow-through was discarded. The spin column was then transferred to another new RNase-free 1.5ml microcentrifuge tube, 100µl of Buffer OEB (at 70°C) was added to resuspend the resin, and then centrifuged for 1 min at maximum speed. The pellet was resuspended with another 100µl of Buffer OEB and centrifuged at maximum speed for 1 min. The Poly A+ mRNA was then stored at -70°C.

2.5 Agarose Gel Electrophoresis

Materials

Balance (accurate to milligrams); microwave oven; power pack and leads; gel tank, gel cast and comb; Fisherbrand pipettors and filter tips (Fisher); glass/pyrex bottle; masking tape; electrophoresis grade agarose gel (Invitrogen); 1× T.B.E. (Tris-Boric Acid-EDTA) Buffer (Invitrogen); 1kb DNA Ladder (Invitrogen); 5mg/ml ethidium bromide (Sigma).

Method

Both ends of the gel cast were sealed using masking tape. 1.200g of agarose was weighed out and transferred into a glass bottle, and 100ml of 1× TBE buffer was added. The bottle was covered with cling film and the solution was heated in the microwave oven until all of the agarose had dissolved. 5µl of ethidium bromide was added, and the solution was mixed well. Once sufficiently cooled, the gel solution was poured gently into the gel cast, and the comb was placed into position. The gel was allowed to set. The samples to be run on the gel were prepared, adding loading dye equalling $1/5^{th}$ total sample volume, and mixed well. When the gel had set, the masking tape was removed from the cast and the cast was placed into the gel tank (pre-filled with 1× TBE buffer. The comb was then removed, and the samples were carefully pipetted into the wells, alongside a 1kb DNA ladder. The voltage was set at 60V for two hours, and switch on to run.

2.6 Polymerase Chain Reaction (PCR) for Sox9a study

Homologous primers for the *Sox9a* gene – based on known sequences from Rainbow trout *Sox9a* (Takamatsu et al., 1997) – were acquired from *Oswel Ltd.*, as were homologous primers for *Actin*, which was chosen as a control to run alongside the *Sox9a* reactions, due to the fact that it is an ancient sequence, and is highly conserved in all eukaryotes.

A PCR protocol and profile were designed, based on work done on salmonids by Woram et. al (2003), and eventually optimised for the DNA, reagents, and laboratory equipment used in the current study. The resultant PCR products were then separated using agarose gel electrophoresis (see above), stained with ethidium bromide, and visualised using a UV Gel-doc system for analysis.

Materials

Eppendorff Mastercycler Gradient thermal cycler (Eppendorff); 0.5ml PCR tubes (Fisher); Fisherbrand pipettors and filter tips (Fisher); nitrile latex-free gloves (Fisher); DNA(se)/RNA(se)-free H_2O (Sigma); genomic Atlantic salmon DNA; PCR primers (Oswel); dNTPs (Invitrogen); *Taq polymerase enzyme (Sigma); *Taq polymerase enzyme buffer solution (Sigma) ; *$MgCl_2$ solution (Sigma)

*Supplied with *RedTaq DNA Polymerase*, Sigma

Method

The PCR reaction mixture was made up in a 0.5ml PCR tube, comprising 0.7µl H_2O; 1.0µl genomic DNA; 2.5µl Sox9a I or Actin I primer; 2.5µl Sox9a II or Actin II

primer; 1.0µl each dNTPs (total 4.0µl dNTPs); 2.0µl Taq buffer; 1.0µl Taq polymerase. Samples were then placed into the thermal cycler and run on the following profile: initial denaturing phase – 5min at 94°C; then 30 cycles of 94˚C for 40s, 55˚C for 50s, 72˚C for 1min 10s; final extension phase - 72°C for 10 minutes. The product was then stored at -20°C until required for further use.

2.7 Polymerase Chain Reaction (PCR) for salmonid genetic markers study

The primers used in this study were as follows:

One102ADFG: (F): CATGGAGAAAAGACCAATCA

(R): TCACTGCCCTACAACAGAAG

Olsen et al., 2000

Ssa406UoS: (F): ACCAACCTGCACATGTCTTCTATG

(R): GCTGCCGCCTGTTGTCTCTTT

Cairney et al., 2000

One18ASC: (F): AGAAACATGAGAACAGTCTAGGT

(R): CCTTATGAGTTTGGTCTCCATGT

Scribner et al., 1996

Sal1UoG: (F): AATGAGCACGTGACCTAGCC

(R): CAGGGTCACACAGAGACACC

Danzmann, R., pers. comm

OmyFGT8TUF: (F): AAGTGTTGGCCTCAGACCTG

(R): GAGCTCCCTCCTCAGAATACC

Danzmann, R., pers. comm.

Omy11INRA: (F): CAACGGACATTTCATTGG

(R): GGTGTTTATTGGGCTAAAGA

Danzmann, R., pers. comm.

Str4INRA: (F): AGCCGATGTATCAGTCACC

(R): CCTAACTGACCTGAGACAGGG

Danzmann, R., pers. comm.

All primers were synthesised by *Invitrogen* custom oligonucleotides, United Kingdom.

Materials

Eppendorff Mastercycler Gradient thermal cycler (*Eppendorff*); 0.5ml PCR tubes (Fisher); Fisherbrand pipettors and filter tips (Fisher); nitrile latex-free gloves (Fisher); DNA(se)/RNA(se)-free H_2O (Sigma); genomic Atlantic salmon DNA; PCR primers (Invitrogen); dNTPs (Sigma); *Taq polymerase enzyme (Sigma); *Taq polymerase enzyme buffer solution (Sigma) ; *$MgCl_2$ solution (Sigma)

*Supplied with *RedTaq DNA Polymerase*, Sigma

Method

20µl PCR reaction mixtures were made up, comprising 10ng (approx.) genomic Atlantic salmon DNA; 10× PCR reaction buffer (1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl at pH 8.3); 200µM dNTPs; 0.15µM each of forward and reverse primer; 0.05 units Taq Polymerase. The samples were then placed into the thermal cycler and run of the following profile: initial denaturation – 5 min at 95°C; then 36 cycles of 0.5 min at 95°C, 1 min at 56°C, 1 min at 72°C; followed by a final extension step of 10 min at 72°C. The PCR products were then separated on a 1.2% agarose gel, containing 1x TBE and 0.5µg/ml ethidium bromide. The fragments were then visualised using a UV trans-illuminator (*Fisher Scientific*).

2.8 Suppressive Subtractive Hybridisation (SSH)

2.8.1 General equipment

1.5ml microcentrifuge tubes (Fisher); Geneamp 2400 thermal cycler (GeneAmp); water bath (Grant); microcentrifuge (Eppendorf); air incubator (Memmert); pipettes and filter-tips (Gilson); vortex (Fisherbrand); nitrile powder-free gloves (Fisher). All reagents for suppressive subtractive hybridisation supplied with *PCR-Select cDNA Subtraction Kit*, Clontech unless otherwise stated.

2.8.2 First-strand cDNA synthesis:

Materials

Sterile H_2O (Sigma); Poly A^+ RNA (self-extracted); skeletal muscle cDNA (self-extracted); 10µM cDNA synthesis primer; 10mM dNTP mix; 20 units/µl AMV Reverse Transcriptase; 5x First-strand buffer (containing: 250mM Tris-HCl, pH 8.5; 40mM $MgCl_2$; 150mM KCl; 5mM Dithiothreitol)

Method

This protocol was followed for each sample of PolyA^+ RNA (male and female Atlantic salmon Poly A^+ RNA), and with the Control PolyA^+ RNA provided in the kit. The skeletal muscle cDNA made here serves as control driver cDNA later on.

Three reaction mixtures were made up, one each for male, female, and control skeletal muscle PolyA$^+$ RNA in sterile 0.5ml microcentrifuge tubes, each comprising 4µl (~2µg) PolyA$^+$ RNA and 1µl of 10µM cDNA Synthesis Primer. Each mixture was then mixed well and incubated at 70°C for 2 min in a thermal cycler. They were then cooled on ice for 2 min, and briefly centrifuged. Added to each reaction was 2µl of 5× First-strand buffer; 1µl of 10mM dNTP mix; 1µl sterile H_2O; 1µl of 20units/µl AMV reverse transcriptase to make the reaction mixture up to 10µl. The mixture was then vortexed gently and briefly centrifuged. The tubes were then incubated at 42°C for 1.5hr in an air incubator, and placed on ice ready for the second-strand cDNA synthesis stage.

2.8.3 Second-strand cDNA synthesis

Materials

5x Second-strand buffer (500mM KCl; 50mM ammonium sulphate; 25mM $MgCl_2$; 0.75mM β-NAD; 100mM Tris-HCl, pH 7.5; 0.25mg/ml BSA); 20x Second-Strand Enzyme Cocktail (6 units/µl DNA Polymerase I; 0.25 units/µl RNase H; 1.2 units/µl *E. coli* DNA ligase) ; 20x EDTA/Glycogen Mix (0.2M EDTA; 1mg/ml Glycogen); 3 units/µl T4 DNA Polymerase; Phenol:Chloroform:Isoamylalcohol (Sigma); Chloroform:Isoamylalcohol (Sigma); 4M ammonium acetate; pure ethanol (Fisher).

Method

48.4µl of sterile H_2O; 16.0µl of 5× second-strand buffer; 1.6µl of 10mM dNTP mix; and 4.0µl of 20× second-strand enzyme cocktail was added to each of the samples of first-strand cDNA synthesised in section 2.8.2, making a final reaction volume of 80µl. The mixture was then mixed thoroughly and briefly centrifuged. The samples were then incubated at 16°C for 2 hours in a thermal cycler. After incubation, 2µl (6 units) of T4 DNA Polymerase was added to each sample, mixed, and incubated at 16°C for 30 min in a thermal cycler. 4µl of 20× EDTA/Glycogen mix was then added to terminate second-strand synthesis. 100µl of phenol:chloroform:isoamlyalcohol was added, and the mixture was vortexed, then centrifuged at 14,000rpm for 10min at room temperature. The top aqueous layer was collected and transferred into a fresh 0.5ml microcentrifuge tube. The intermediate and lower phases were discarded. 100µl of chloroform:isoamylalcohol was added to the transferred upper phase, vortexed, and centrifuged at 14,000rpm for 10min at room temperature. The top aqueous layer was transferred into a fresh 0.5ml microcentrifuge tube, and the intermediate lower phases

were discarded. 40µl of 4M ammonium acetate and 300µl of 95% ethanol was added to the newly transferred upper aqueous phase, vortexed, and centrifuged at 14,000rpm for 20min at room temperature. The supernatant was carefully removed, and the remaining pellet was resuspended in 500µl of 80% ethanol. The tube was then centrifuged at 14,000rpm for 10min, and the supernatant was removed. The tube was left to air dry to allow residual ethanol to evaporate away. The final precipitate was resuspended in 50µl of sterile H_2O. 6µl of the cDNA was transferred to a fresh microcentrifuge tube and stored at -20°C until after *Rsa* I digestion phase (section 2.8.4).

2.8.4 *Rsa* I digestion

Materials

Experimental tester and driver ds cDNA (from section 2.8.3); control skeletal muscle cDNA (from section 2.8.3); 10× *Rsa* I restriction buffer (100mM bis tris propane-HCl, pH 7.0; 100mM $MgCl_2$; 1mM dithiothreitol); 10 units/µl *Rsa* I; 20× EDTA/Glycogen Mix; Phenol:Chloroform:Isoamylalcohol (Sigma); Chloroform:Isoamylalcohol (Sigma); 4M ammonium acetate (Sigma); pure ethanol (Fisher).

Method

Three reaction mixtures were made up, using each of the ds cDNA samples synthesised in section 2.8.3. Each mixture contained 43.5µl of ds cDNA; 5.0µl of 10× *Rsa* I Restriction Buffer; 1.5 µl of *Rsa* I (10 units/µl). The tubes were then vortexed, briefly centrifuged, and incubated at 37°C for 1.5 hours. 5µl of the digestion mixture was set aside for later analysis of *Rsa* I digestion efficiency. 2.5µl of 20× EDTA/Glycogen mix and 50µl of phenol:chloroform:isoamylalcohol was added to the remaining digest, then vortexed and centrifuged at 14,000rpm for 10 min at room temperature. The upper aqueous layers were collected and transferred into fresh 0.5ml microcentrifuge tubes. 50µl of chloroform:isoamylalcohol was added, and the mixtures were vortexed and centrifuged at 14,000rpm for 10 min at room temperature. The upper aqueous layers were collected and transferred into further 0.5ml microcentrifuge tubes. 25µl of 4M ammonium acetate and 187.5µl of 95% ethanol was added to each of the newly transferred upper aqueous phases, vortexed, and centrifuged at 14,000rpm for 10 min at room temperature. The supernatant was removed from each tube, the pellets were resuspended in 200µl of 80% ethanol, and

then centrifuged at 14,000rpm for 5 min. The supernatant was then removed again and the pellets were dried in air for 10 min. The pellets were then resuspended in 5.5µl of H_2O and stored at -20°C.

These 5.5µl samples of *Rsa* I digested cDNA will serve as the experimental driver cDNA and the control skeletal muscle driver cDNA. The sample of *Rsa* I –digested cDNAset aside after the digestion step was then separated on a 2% agarose/EtBr gel, to check the completion of the digestion.

2.8.5 Adaptor Ligation

In this stage, the *Rsa* I digested cDNA samples (section 2.8.4) are ligated with adaptors to create the tester cDNAs for forward, control, and reverse subtractions.

Materials

Rsa I-digested **experimental** cDNA (from section 2.8.4); control skeletal muscle cDNA; sterile H_2O (Sigma); 5x Ligation Buffer (250 mM Tris-HCl, pH 7.8; 50mM $MgCl_2$; 10mM dithiothreitol; 0.25 mg/ml BSA); T4 DNA Ligase (400 units/µl; containing 3mM ATP); diluted tester cDNA; 10µM Adaptor 1; 10µM Adaptor 2R; 20x EDTA/Glycogen Mix.

Method

1µl of each *Rsa* I-digested **experimental** cDNA was diluted with 5µl of sterile H_2O and labelled'diluted tester cDNA'. A ligation master mix was then prepared in a 0.5ml microcentrifuge tube, comprising 15µl sterile H_2O; 10µl of 5× ligation buffer; 5µl of T4 DNA ligase (400 units/µl). Four 10µl ligation reaction samples were then prepared and labelled ♂-1, ♂-2, ♀-1, ♀-2, each consisting of the following:

	♂-1	**♂-2**	**♀-1**	**♀-2**
Diluted tester cDNA	2µl	2µl	2µl	2µl
Adaptor 1	2µl	0	2µl	0
Adaptor 2R	0	2µl	0	2µl
Master Mix	6µl	6µl	6µl	6µl

The samples were then centrifuged briefly, and incubated at 16°C overnight. 1µl of EDTA/Glycogen mix was added to stop the ligation reaction, and the samples were heated at 72°C for 5min to inactivate the ligase. The tubes were then briefly centrifuged and stored at -20°C.

2.8.6 First Hybridisation

In this procedure, an excess of driver cDNA is added to each tester cDNA, samples are heat denatured, and allowed to anneal. The remaining ss cDNAs (available for second hybridisation) are dramatically enriched for differentially expressed sequences because non-target cDNAs present in the tester and driver cDNA form hybrids.

Materials

♂ Undiluted *Rsa* I-digested Driver cDNA; ♀ Undiluted *Rsa* I-digested Driver cDNA; Adaptor 1-ligated Tester ♂-1; Adaptor 2R-ligated Tester ♂-2; Adaptor 1-ligated Tester ♀-1; Adaptor 2R-ligated Tester ♀-2; 4x Hybridisation buffer.

Method

The 4× hybridisation buffer was incubated at 37°C for 10 min. 4.0µl samples for the first hybridisation (labelled ♂-1 +(D), ♂-2 +(D), ♀-1 +(D), ♀-2 +(D)) were then prepared in 0.5ml tubes, consisting of the following:

	♂-1 +(D)	**♂-2 +(D)**	**♀-1 +(D)**	**♀-2 +(D)**
♀ *Rsa* I-digested driver cDNA (undiluted)	1.5 µl	1.5 µl	0	0
♂ *Rsa* I-digested driver cDNA (undiluted)	0	0	1.5 µl	1.5 µl
Adaptor 1-ligated Tester ♂-1	1.5 µl	0	0	0
Adaptor 2R-ligated Tester ♂-2	0	1.5 µl	0	0
Adaptor 1-ligated Tester ♀-1	0	0	1.5 µl	0
Adaptor 2R-ligated Tester ♀-2	0	0	0	1.5 µl
4x Hybridisation Buffer	1 µl	1 µl	1 µl	1 µl

The samples were then centrifuged briefly and incubated at 98°C for 1.5min in a thermal cycler, then at 68°C for 8 hours.

2.8.7 Second Hybridisation

The two samples from the first hybridisation are mixed together, and fresh denatured driver DNA is added to further enrich for differentially expressed sequences. New hybrid molecules are formed which consist of differentially expressed cDNAs with different adaptors on each end. It is important that the samples from the first hybridisation are not denatured, and that the samples are not removed from the thermal cycler for longer than is necessary to add fresh driver.

Materials

First hybridisation samples ♂-1 +(D), ♂-2 +(D), ♀-1 +(D), ♀-2 +(D); fresh driver cDNA (from section 2.8.4); 4× Hybridisation Buffer; sterile H_2O.

Method

"♀ driver" for ♂ hybridisation was prepared in a fresh 0.5ml microcentrifuge tube, comprising: 0.75µl of undiluted ♀ *Rsa* I-digested cDNA (from section 2.8.4, *Rsa* I Digestion) and 0.25µl 4x Hybridisation Buffer. "♂ driver" for ♀ hybridisation was prepared in another fresh tube, comprising: 0.75µl Undiluted ♂ *Rsa* I-digested cDNA (from section 2.8.4, *Rsa* I Digestion) and 0.25µl 4x Hybridisation Buffer. Both driver samples were then vortexed, briefly centrifuged, and incubated at 98°C for 1.5min in a thermal cycler. The tubes were then removed from the thermal cycler.

A micropipettor was set at 15µl, and first hybridisation sample ♂-2+(D) was drawn into the pipettor tip, with a little air. The freshly denatured ♀ driver was then also drawn into the same pipettor tip, and transferred alongside sample ♂-2+(D) into the

tube containing first hybridisation sample ♂-1+(D). The mixture was pipetted up and down thoroughly to mix. With a fresh pipettor tip, first hybridisation sample ♀-1+(D) was drawn into the pipettor tip, with a little air. The freshly denatured ♂ driver was then also drawn into the same pipettor tip, and transferred alongside sample ♀-1+(D) into the tube containing first hybridisation sample ♀-2+(D). The mixture was pipetted up and down thoroughly to mix. Both reaction samples were then briefly centrifuged and incubated at 68°C in a thermal cycler overnight. 200µl of dilution buffer was then added to both samples and mixed by pipetting. The samples were incubated at 68°C for 7min in a thermal cycler, briefly centrifuged, then stored at -20°C.

2.8.8 Primary PCR Amplification

Differentially expressed cDNAs are selectively amplified during the two reactions described in this section. Before thermal cycling, the missing strands of the adaptors are filled in by a brief incubation at 75°C. This creates the binding site for PCR Primer 1. In the first amplification, only ds cDNAs with different adaptor sequences on each end are exponentially amplified. In the second amplification, nested PCR is used to further reduce background and enrich for differentially expressed sequences.

Materials

Diluted cDNA; unsubtracted cDNA; subtracted cDNA; sterile H_2O; 10× PCR Reaction Buffer; 10mM dNTP mix; 10mM PCR Primer 1; 50× Advantage cDNA Polymerase Mix.

Method

Primary PCR reaction samples were prepared in PCR tubes labelled "♂", "♀", and "–ve control", comprising of the following: for ♂, 19.5µl sterile H_2O; 2.5µl 10× PCR reaction buffer; 0.5µl 10mM dNTP mix; 1.0µl 10mM PCR primer 1; 0.5µl 50× Advantage cDNA polymerase mix; 1.0µl ♂ hybridised cDNA (from section 2.8.7), for ♀: 19.5µl sterile H_2O; 2.5µl 10× PCR reaction buffer; 0.5µl 10mM dNTP mix; 1.0µl 10mM PCR primer 1; 0.5µl 50× Advantage cDNA polymerase mix; 1.0µl ♀ hybridised cDNA (from section 2.8.7), and for –ve control: 20.5µl sterile H_2O; 2.5µl 10× PCR reaction buffer; 0.5µl 10mM dNTP mix; 1.0µl 10mM PCR primer 1; 0.5µl 50× Advantage cDNA polymerase mix.

Each sample was vortexed, briefly centrifuged, and incubated at 75°C for 5min in a thermal cycler, then run on the following profile: 94°C for 25 sec, then 27 cycles of 94°C for 10 sec; 66°C for 30 sec; 72°C for 1min 30sec. 8µl of each sample was aliquoted from each reaction, 1.7µl loading buffer was added, and these tubes were stored at -20°C. A further 3µl of each reaction was then diluted in 27µl of H_2O and stored at -20°C.

2.8.9 Secondary PCR Amplification

Materials

Diluted PCR product from primary amplification; sterile H_2O (Sigma); 10x PCR Reaction Buffer; 10mM Nested PCR Primer 1; 10mM Nested PCR Primer 2R; 10mM dNTP Mix; 50x Advantage cDNA Polymerase Mix.

Method

A master mix for the secondary PCR reactions was prepared, consisting of 74µl sterile H_2O; 10µl 10× reaction buffer; 4µl Nested PCR primer 1; 4µl Nested PCR primer 2R; 2µl dNTP mix; 2µl 50× Advantage cDNA polymerase mix. The mixture was vortexed and briefly centrifuged, then 24µl was aliquoted into each of three new tubes, labelled "♂", "♀", and "-ve". 1µl of each corresponding diluted primary PCR product (section 2.8.8) was added to the three aliquots of master mix, vortexed, and briefly centrifuged. The tubes were then placed into a thermal cycler and run on the following profile for 12 cycles: 94°C for 10sec, 68°C for 30sec, 72°C for 1min 30sec. 8µl of each reaction was then analysed alongside the primary PCR reaction products (from 2.8.8) on a 2.0% agarose/EtBr gel run in 1× TBE buffer . The remaining reaction product was stored at -20°C.

3. *Sox9a*

Presented here is a narrative of, and findings from, work done in relation to the *Sox9a* gene. Included is the rationale behind the work, the challenges and difficulties faced whilst undertaking the work, and the context in which the work is set.

The fish used for this part of the current study were Atlantic salmon parr (juveniles) supplied by the Cynrig Hatchery, on the Cynrig River – a tributary for the River Usk near Brecon in Wales, United Kingdom. The animals were anaesthetised on capture, packed in dry ice, and transported by road to the University of Portsmouth laboratories, where they were stored at < -20°C until required for use. The tissue for the study was required to come from the adipose fin of the Atlantic salmon. The reason for using the adipose fin as opposed to any other part of the anatomy is that it can be removed without harming the animal, and does not affect its normal function, nor the stability of the fish in the water, thus satisfying the non-destructive criterion of the ultimate aim: the development of a non-destructive tool for sexing juvenile Atlantic salmon.

The following sections describe the optimisation of protocols to extract and purify DNA from adipose fin tissue; optimisation of PCR protocols; and the results of this strand of work.

3.1 Optimisation of DNA extraction and purification methods

According to the initial aims and requirements of the study, it was important to develop methods to extract and purify DNA from wild samples that were of high enough quality to be suitable for use in PCR amplification.

The method used for tissue digestion in the current study (see section 2.1) is based on a protocol for obtaining DNA from salmonid fish by Spruell & Thorgaard (1996). The method was modified to reduce DNA degradation by reducing the incubation temperature from 60°C to 37°C, and conditions were controlled, using a covered water-bath so that potential damage to the DNA from UV-light sources (although low-risk) were eliminated.

The method used for the extraction of DNA (see section 2.2) in the current study is also based on well-established molecular biology techniques. The method evolved markedly during the optimisation process, with changes being made to centrifugation times (to accommodate for extra waste tissue); cooling times (for precipitation of proteins); and reagents used (substituting one chloroform:isoamylalcohol extraction for a phenol:chloroform:isoamyalcohol extraction). Despite the risks of phenol actually damaging the DNA, it denatures proteins well and it is thought that this improved the overall purity of the DNA yielded.

The resultant stock, following optimisation of the extraction and purification protocols, was of molecular biology-grade DNA, suitable for investigative work. However, despite being the best it could be, given the parameters under which the

work was carried out, the quality of the yielded DNA was by no means ideal. Using agarose gel electrophoresis (see section 2.7) to analyse the DNA, significant 'streaking' can be observed on the gel when viewed using a gel documentation system (see Fig. 3.1.1). Despite reducing the concentration of genomic DNA used in subsequent experiments, this 'streaking' remained difficult to eliminate.

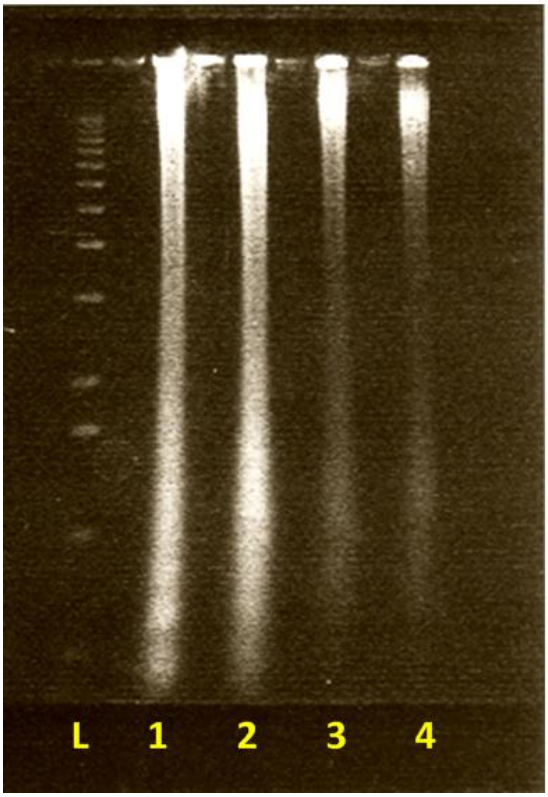


Fig 3.1.1

Agarose gel showing 4 initial samples of extracted Atlantic salmon DNA (1-4), alongside a 1kb DNA ladder (L). Note the extensive 'streaking' that can be seen in all four samples. The greater amount of genetic material that appears to be present in lanes 1-2 is due to a greater amount of tissue used.

The DNA was run on a 1.2% agarose gel containing 5mg/ml ethidium bromide at 15V overnight, alongside a 1kb DNA ladder (L). Lanes 1-4 contain DNA obtained from adipose fin tissue from four male Atlantic salmon. Calculated yields from a 0.5cm^2 adipose fin sample vary from 0.74-1.56µg of genomic DNA.

3.2 Optimisation of Polymerase Chain Reaction (PCR) methods

With a working stock of Atlantic salmon DNA of suitable quality available, the next step was to optimise the PCR protocols. Initial tests involved using *Hpa* I primers, to test the viability of the genomic DNA for use in PCR.

With PCR being a notoriously sensitive process, and with so many variables involved in the technique, protocol optimisation can be, and indeed proved to be, a time-consuming process.

On the eventual successful completion of initial tests (thus, confirming the suitability of the extracted genomic DNA for investigative PCR work), the *Sox9a* gene was selected for testing. This represents a strategy that can be likened to that of the 'low-hanging fruit' – one that is arguably the 'easy' choice, with the hope that the pay-off is a positive one. The aim of this study is to develop a molecular technique for positive identification of gender in juvenile Atlantic salmon. Given the importance of the *SoxE* (*Sox8, 9* and *10*) genes in testis development and proliferation of spermatozoa in vertebrates, perhaps it is not too far-fetched an idea that they play a key gender-determining role in the species in question. Review of the available literature suggests that *Sox9* in particular has a key role in testis development, and work by Takamatsu et al. (1997) suggested that *Sox9a* may play a role in sex determination and differentiation in fish, due to its prominent expression in rainbow trout testis. With rainbow trout being closely related to Atlantic salmon, *Sox9a* was determined to be the ideal place to begin investigations.

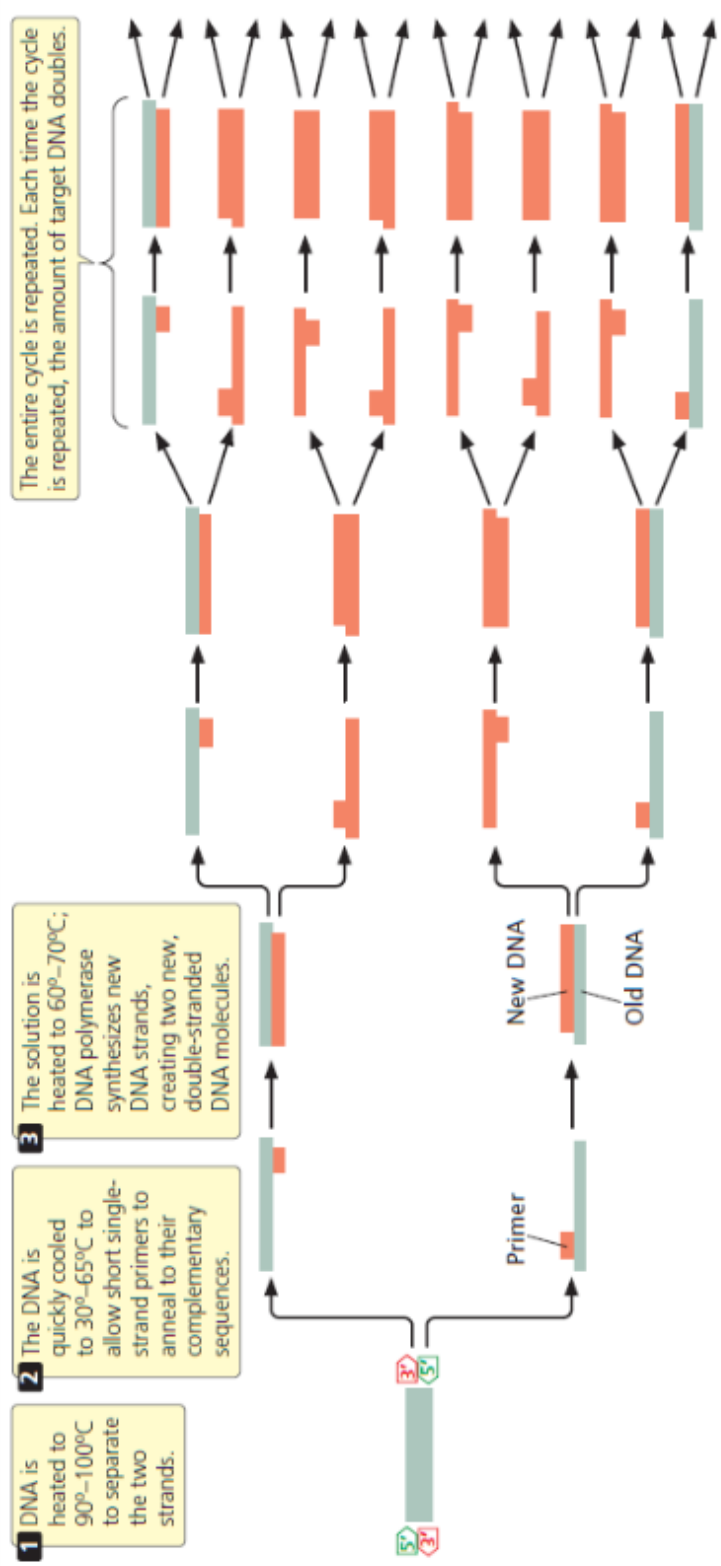


Fig. 3.2.1

Diagrammatic representation of the PCR process.

(from Genetics: A Conceptual Approach by Benjamin A Pierce, 2002)

3.3 Results

It is clear that the *Sox9a* gene is present in the genomic DNA of male individuals in this population of Atlantic salmon (see Fig. 3.3.1).

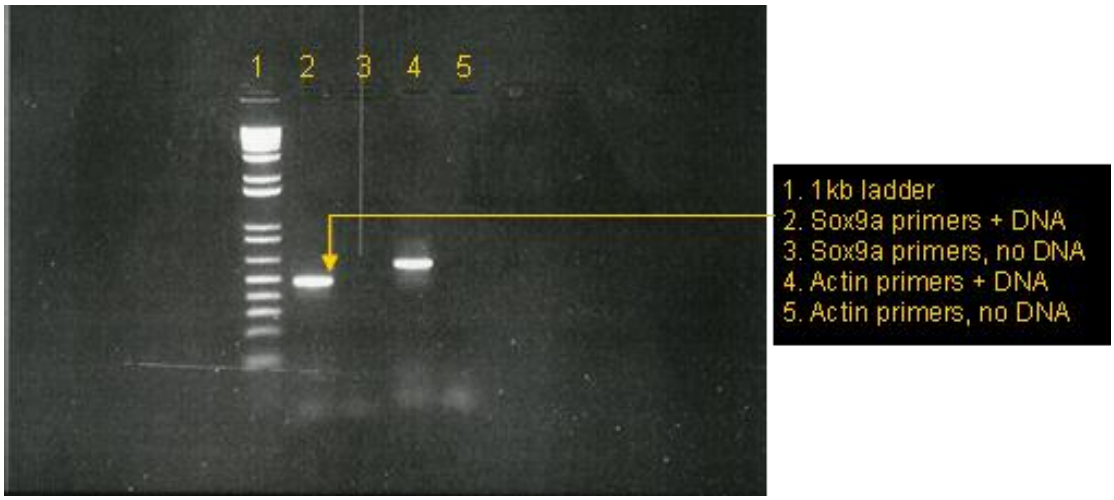


Fig. 3.3.1

PCR products of *Sox9a* and *Actin* (control) primers using DNA extracted from a male individual of the Cynrig population of Atlantic salmon, showing bright bands at 500bp (*Sox9a*) and at ~600bp (*Actin*), thus confirming that the *Sox9a* gene is present in this male individual of the River Usk population of Atlantic salmon.

On confirmation that *Sox9a* is expressed in River Usk male Atlantic salmon, the next step was to determine whether the gene is expressed in the corresponding female population. The same process was followed, using DNA from a female individual of the same population of salmon as the original male template genomic DNA, and

results show that *Sox9a* is also found to be present in the female complement of River Usk Atlantic salmon (Fig. 3.3.2).

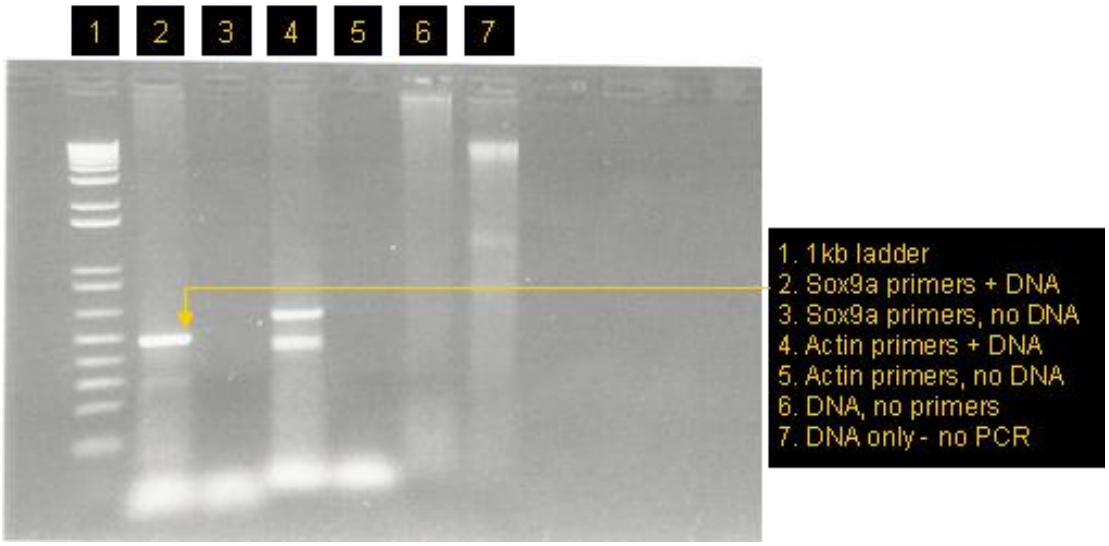


Fig. 3.3.2

PCR products of *Sox9a* and *Actin* (control) primers using DNA extracted from a female individual of the River Usk population Atlantic salmon, showing bright bands at 500bp (*Sox9a*) and at 600bp (*Actin*), thus confirming that the *Sox9a* gene is present in this female individual of the River Usk population of Atlantic salmon. Note the secondary band at ~500bp in track 4.

It can be taken from the two tests, that the *Sox9a* gene is present in both the male and female complements of this population of Atlantic salmon, and therefore rules out any possibility of this gene being a gender-determining factor.

Under normal circumstances, this outcome would effectively terminate the strand of research being undertaken. However, on further analysis of this gel (Fig. 3.3.2 – the

gel relating to the use of female template DNA) – it is clear that there is a secondary band (and possibly a tertiary band very close to it), in the lane containing the PCR product of the reaction involving the *Actin* primers. This (or indeed, these) band(s) are not seen at all in the gel relating to male template DNA, so it was deemed necessary to carry out further investigations. The two experiments were run again, this time concurrently, with the PCR products being separated on the same gel (Fig. 3.3.3). The findings confirmed the two previous observations, these being:

1. The products of the reactions using the *Sox9a* primers are identical, whether using male or female template DNA (tracks 2 and 3).
2. There are definite differences between the PCR products of the *Actin* reactions (tracks 6 and 7, circled).

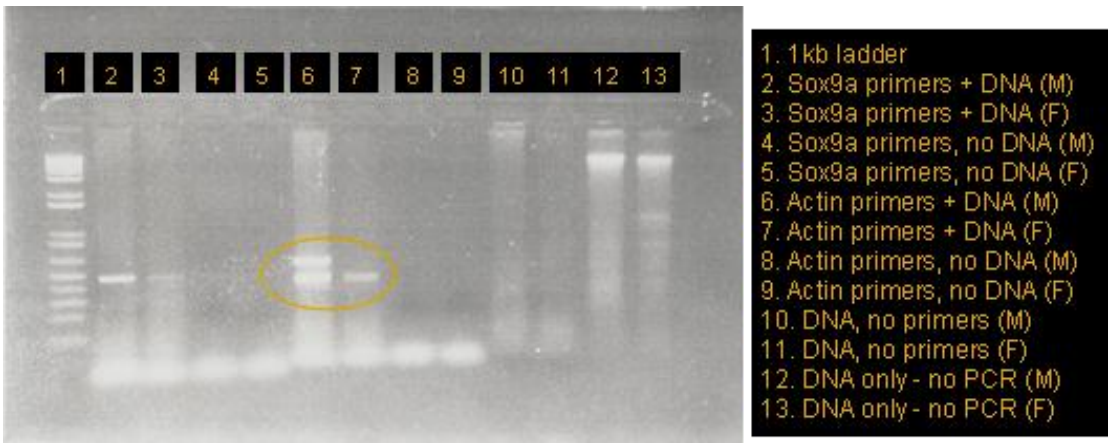


Fig. 3.3.3

PCR products of the reaction between homologous *Sox9a* and *Actin* primers, with DNA extracted from a male and a female individual of the River Usk population of Atlantic salmon.
Identical bands can be seen in the male and female products of the reaction with *Sox9a* primers; and a different banding pattern in male and female products of the reaction with *Actin* primers (circled).

However, the multiple bands appeared this time in the male template DNA, and not in the female template DNA. This was a reversal of the findings in the previous experiments. In an attempt to quantify this curious outcome, PCR reactions were set up using *Actin* primers ONLY, and using DNA from three separate males, and three separate females (Fig. 3.3.4).

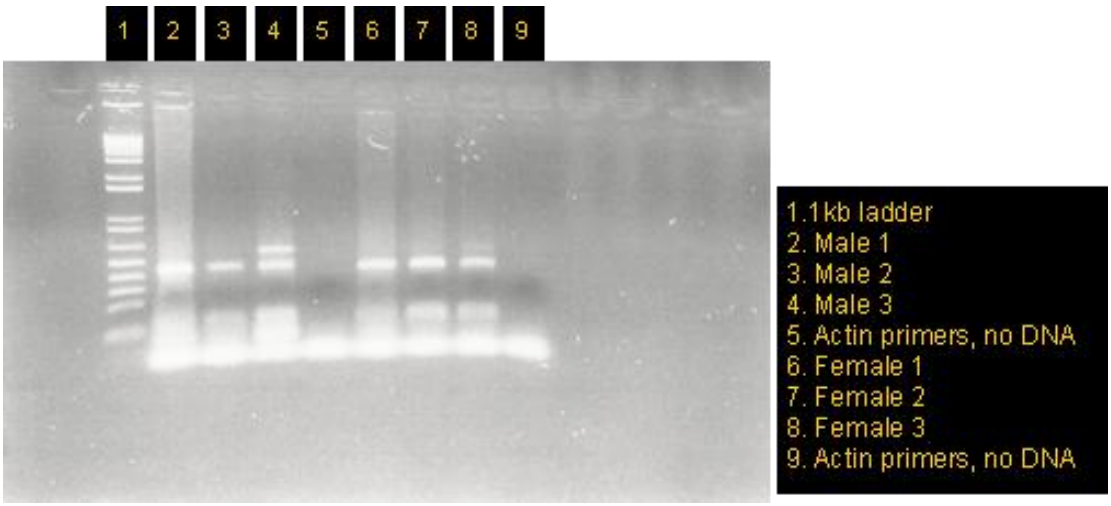


Fig. 3.3.4

PCR products of the reaction between homologous *Actin* primers and DNA extracted from three male and three female individuals of the River Usk population of Atlantic salmon.
In all samples, there is a clear band at around 450bp, but there are secondary, tertiary, and possibly further bands in several of the tracks (most notably, in tracks 3, 4, 7, 8).

Although a common band can be seen on the gel, corresponding to the bands for *Actin* in Fig. 3.3.1 and Fig. 3.3.2, several of the samples show banding patterns with

evidence of secondary, tertiary, and even higher-multiple bands. On repetition of this experiment, different results still can be observed (see Fig. 3.3.5).

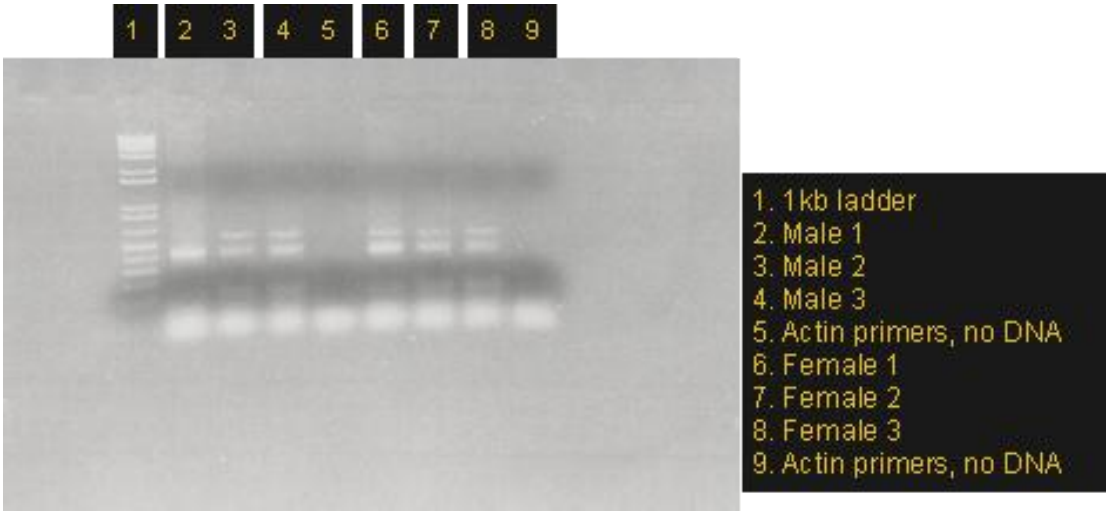


Fig. 3.3.5

PCR products of the reaction between homologous *Actin* primers and DNA extracted from three male and three female individuals of the Cynrig population of Atlantic salmon.

Note the differing banding pattern in this gel compared to Fig. 3.7, despite being a repetition of the same experiment.

3.4 Discussion

The optimisation of techniques for the extraction and purification of molecular grade DNA from wild tissue was a challenging process. Despite the difficulties faced, the resulting DNA was of suitable quality to commence further investigative work, and results were obtained from these tests. Although not a formal part of the aims and objectives of the current study, it is worth mentioning the curious case of the differential patterns observed in the actin bands of the gels. From the evidence shown

in this data, it is possible to deduce that there is a degree of polymorphism within the *Actin* genes of the Atlantic salmon, but this does not appear to have any influence on gender, and therefore nor on the mechanisms thereof. Despite being interesting observations that may merit further investigation in a scientific context, it is beyond the scope of the current study to pursue this strand of research any further, and so further work to investigate these observations in the PCR products of *Actin* was terminated.

In answer to the question asked in this investigation – namely, 'Is the *Sox9a* gene gender-specific in Atlantic salmon, and if so, can it be used as a sex-determining factor in this species?' the answer must be NO on both counts. Having used primers designed to bind to specific sequences in the DNA of the Atlantic salmon, and having amplified those sequences by PCR to a magnitude where it is possible to analyse through visual means, the presence of a band in the PCR product of the reaction between the *Sox9a* primers and both male and female template DNA is strong evidence that the *Sox9a* gene is present in both genders, and thus *Sox9a* is NOT gender-specific, and certainly CANNOT be used as a sex-determining factor in Atlantic salmon.

Although the requirement of *Sox9* in testis formation, and therefore its nomination as a key factor in sex determination/differentiation in the majority of the vertebrate groups is widely accepted (Kent et al., 1996; Clarkson & Harley, 2002; Barrionuevo & Scherer, 2010), the gene and its various orthologues appear to be expressed in testes (Takamatsu et al., 1997; Nakamoto et al., 2005; Chiang et al., 2001; Zhou et al., 2003) and ovaries (Yokoi et al., 2002; Chiang et al., 2001; Zhou et al., 2003), and in

ovotestes (Zhou et al., 2003) in different species of fish. These data suggest that the role of the *Sox9* gene during gonadal development differs in fish from species to species. For example, data suggests that *Sox9* has a significant role in testicular differentiation in rainbow trout (Vizziano et al., 2007) and tilapia (Ijiri et al., 2008). Conversely, *Sox9a2* (a *Sox9* orthologue) expression in somatic cells during early gonadal differentiation is equal in both males and females in the medaka fish, but is maintained in the male during testicular lobe formation, indicating that although not involved in sex determination, *Sox9* is involved in development of the testicular lobe in medaka (Nakamura et al., 2008). There are two orthologues of *Sox9* in zebrafish, *Sox9a* and *Sox9b*. *Sox9a* is found in the testes (and brain, kidney and muscle), and *Sox9b* can be found in the ovary (Chiang et al., 2001). *Sox9a* mutants show craniofacial malformations and lack of cartilage, which is a condition similar to campomelic dysplasia in humans. Despite this, they are able to reproduce, suggesting that *Sox9a* does not direct sex determination/differentiation in zebrafish (von Hofsten & Olsson, 2005). A recent study on the testis-specific enhancer of *Sox9* (TESCO) revealed an evolutionarily conserved region (ECR) of 180bp that is present in marsupials, monotremes, birds, reptiles, and amphibians, but interestingly, not in fish (Bagheri-Fam et al., 2010). This suggests that tetrapods may share common aspects of *Sox9* regulation, but (due to the absence of the ECR) this is not shared in fish. Even though expression patterns of the *Sox9* gene/s may be similar among some fish species, such as the patterns seen in *Sox9a* expression in the triploid crucian carp and zebrafish (Guo et al., 2010), one can reasonably suggest that the role of *Sox9* and its orthologues in sex determination/differentiation may be different in fish as a group, than in other vertebrates. With such variation in, and lack of consensus regarding, the role of *Sox9* in piscine sex, it becomes rather more difficult to suggest with conviction,

the notion of *Sox9*, or indeed any of its orthologues, as a male-determining factor in fish.

Despite the results presented in the present study suggesting that expression of *Sox9a* is not specific to gender in Atlantic salmon, this does not account for the possibility of different complements and configurations of microRNAs (miRNAs) between the two sexes, leading to differing expression of *Sox9a* or indeed its orthologues, and in a wider context, the *Sox* genes as a whole. In order to quantify this, one approach would be to synthesise primers for every *Sox* gene (and any orthologues), and run PCRs for each, comparing the products of reactions using male DNA with those where female DNA is used. However, this would be immensely time-consuming and besides, there can be no guarantee that any of these sequences are key sex determining factors at all!

At this point, two further directions of study were identified. The first is to explore sequences that have been shown to be gender-specific in various species of the *Oncorhynchus* genus – that is, members of the Pacific salmon family of fish, which are closely related to Atlantic salmon. The second direction involves a much more radical mode of thought, necessitating the removal of all preconceptions one might have about mechanisms of sex determination/differentiation in nature, and characterising the actual differences that exist in the expression patterns of the genomes of male and female Atlantic salmon. These form the basis of further investigative work in the present study.

4. Molecular connotations of Salmon gender

This chapter comprises results and discussion of the remaining experimental work undertaken in the current study, and can be taken as two discrete strands of investigation. The first explores genetic markers that have been putatively linked to gender in various *Oncorhynchus* species, and the aim of this mode of study was to ascertain whether these markers can also be used to assay for gender in Atlantic salmon. The second mode of study presented here describes a novel approach whereby a suppressive subtraction hybridisation technique is used in an attempt to isolate gender-specific expressed sequence tags (ESTs).

4.1 Using sex-linked genetic markers from Pacific salmon species to assay for gender in Atlantic salmon

In the present study, seven sequences were chosen, based on work done on the putative sex determining region in a number of salmonid species, including Atlantic salmon (Woram et al., 2003). These sequences (*Omy11INRA*, *Sal1UoG*, *One102ADFG*, *Ssa406UoS*, *Str4INRA*, *One18ASC*, and *OmyFGT8TUF*), are linked to the sex determining locus (*SEX*) in Atlantic salmon, and are homologous in brown trout and rainbow trout (Woram et al., 2003). It was desirable in this study to investigate whether any of the alleles identified by these sequences were linked to

gender, and so primers were synthesised, and PCRs were run with DNA from male and female individuals from the River Usk population of Atlantic salmon.

PCRs were then run with DNA from male and female individuals provided by a commercial supplier. These fish were of the MOWI strain of Atlantic salmon, which were introduced for aquaculture in 1969. The origins of this line lie in wild-caught salmon sourced from various rivers (and therefore, races) in Norway. These caught individuals were then used as broodstock to begin the MOWI hybrid strain. A third race of Atlantic salmon was then tested, using tissue samples from mature salmon of known gender, native to the Burrishole River in Ireland.

4.1.2 Results

The data from PCR tests on the River Usk population of Atlantic salmon shows that five of the seven sets of primers amplified sequences in both male and female components of this population. In all cases (as expected) there is evidence of polymorphism. There was one sequence that indicated gender-specific differences between the male and female samples, and one that partially indicated differences. *Omy11INRA* was found in female salmon from the River Usk, but not in males (Figure 4.1.2i).

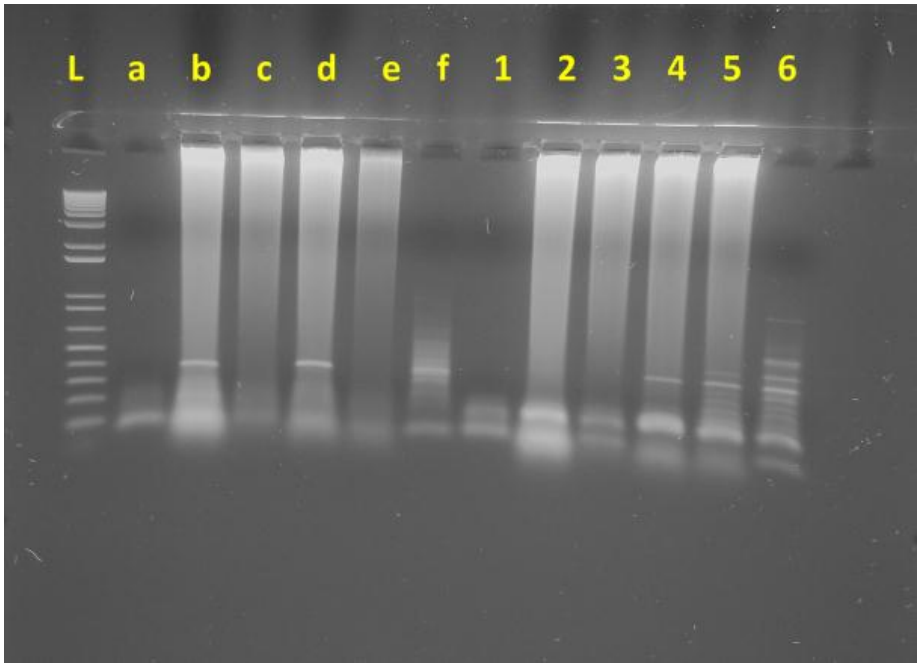


Fig. 4.1.2.i

Agarose gel showing products of PCR reaction using *Str4INRA* (lanes a-f) and *Omy11INRA* (lanes 1-6) primers, run alongside a 1kb ladder (L).

Lanes a, b and c show the PCR products of three males of the River Usk population of Atlantic salmon run with the *Str4INRA* primers, and lanes d, e and f show those of three females from the same population with the same primers. These results show that *Str4INRA* can be found in both male and female Atlantic salmon DNA.

Lanes 1, 2 and 3 show the products of the PCR reaction between the same male samples and the *Omy11INRA* primers. Lanes 4, 5 and 6 represent the products of the reaction using the same primer with the three female DNA samples. It is clear that sequences have been amplified in the female samples, but not in the male samples – note the band common to all female samples found at ~400bp.

In the gel shown in Fig. 4.3.1, the products of the PCR reactions using the *Str4INRA* primers and *Omy11INRA* primers are shown, alongside a 1kb ladder (L). Lanes a, b and c show the PCR products of three males of the River Usk population of Atlantic salmon run with the *Str4INRA* primers, and lanes d, e and f show those of three females from the same population with the same primers. Although bands are not present in all individuals of a particular gender, it is apparent that no differences exist

between genders – that is, the *Str4INRA* sequence can be found in both male and female individuals of this race of Atlantic salmon, and does not therefore appear to be gender-specific. Lanes 1-6 contain the products of the reactions that were run using the *Omy11INRA* primers. Lanes 1, 2 and 3 represent those run using male genomic DNA, and lanes 4, 5 and 6 represent the products of using female genomic DNA. It is clear that sequences have been amplified in the female samples, but not in the male samples. These results were repeated using tissue samples from a total of 15 males and 15 females, each time producing the same results, suggesting that *Omy11INRA* can be used to differentiate between male and female individuals of the River Usk race of Atlantic salmon.

Fig. 4.1.2.ii shows the agarose gel for the products of the PCR reactions using the *Ssa406UoS* and *OmyFGT8TUF* sets of primers. Once again, these products were run alongside a 1kb ladder (L). Lanes a, b and c contain the products from the reactions using the *Ssa406UoS* primers with DNA from three male salmon, and in lanes d, e and f are the products from the reactions using the same primers and DNA from three female salmon. There are no gender-specific differences apparent in the products of these reactions; however once again, the patterns are not uniform among all individuals of the same gender.

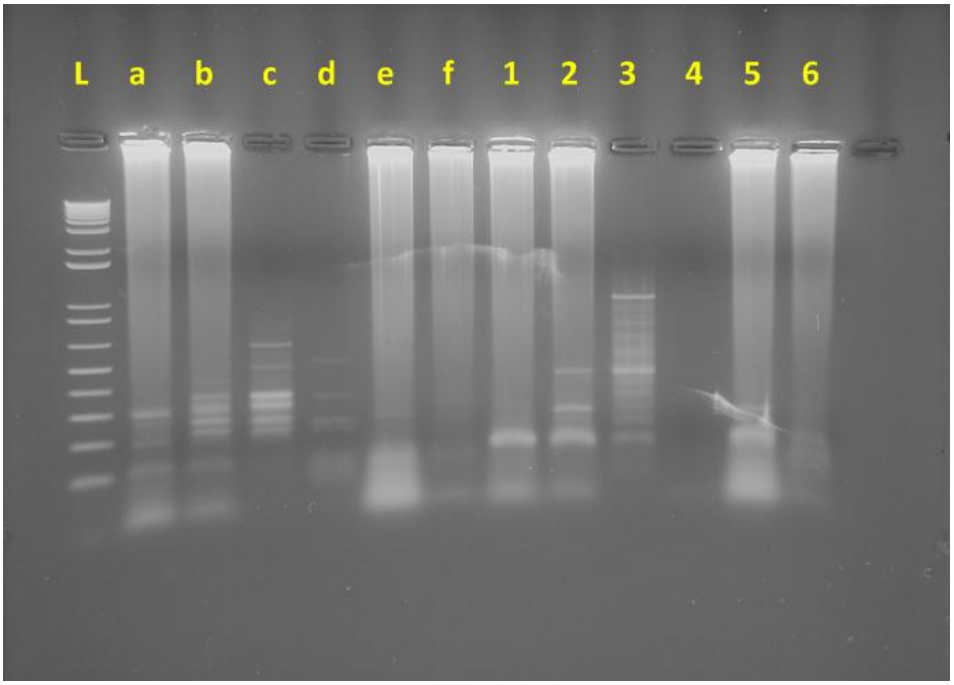


Fig. 4.1.2.ii

Agarose gel showing products of PCR reaction using *SSa406UoS* (lanes a-f) and *OmyFGT8TUF* lanes 1-6) primers, run alongside a 1kb ladder (L). River Usk salmon.

Lanes a, b and c contain the products from the reactions using the Ssa406UoS primers with DNA from three male salmon, and in lanes d, e and f are the products from the reactions using the same primers and DNA from three female salmon. There are no gender-specific differences apparent in the products of these reactions; however once again the patterns are not uniform across all individuals of the same gender.

Lanes 1, 2 and 3 contain the products of reactions using DNA from three male salmon, run with the OmyFGT8TUF primers, and lanes 4, 5 and 6 the PCR products of reactions using DNA from three female salmon with the same primers. There are no amplified products apparent from the reactions run using female DNA, but there are clear bands seen in two of the male samples (lanes 2 and 3), with at least one of these common to both.

Lanes 1, 2 and 3 contain the products of reactions using DNA from three male salmon, run with the *OmyFGT8TUF* primers, and lanes 4, 5 and 6 the PCR products of reactions using DNA from three female salmon with the same primers. There are no amplified products apparent from the reactions run using female DNA, but there

are clear bands seen in two of the male samples, with one of these common to both. When repeated, this band could be seen in most (but not all) of the males tested.

Fig. 4.1.2.iii shows the agarose gels of the products from PCR reactions run with *One102ADFG* and *One18ASC*. The products were run alongside a 1kb ladder (L), and are designated as follows – lanes a-f (*One102ADFG*); lanes 1-6 (*One18ASC*). Male DNA was used in lanes a, b, c, and lanes 1, 2, 3. Female DNA was used in lanes d, e, f, and lanes 4, 5, 6.

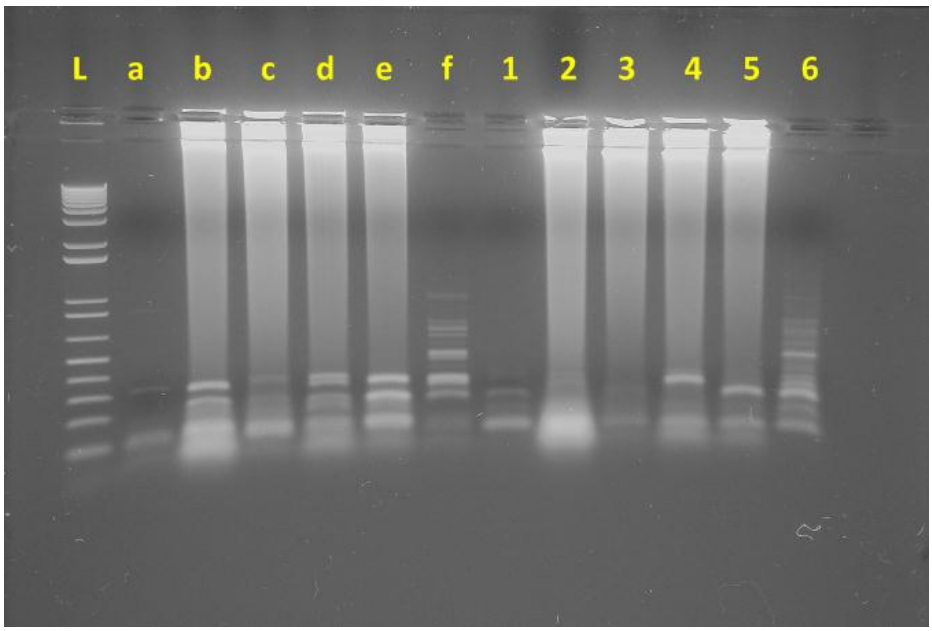


Fig. 4.1.2.iii

Agarose gel showing the products of PCR reaction using *One102ADFG* primers (lanes a-c with male DNA, lanes d-f with female DNA) and *One18ASC* primers (lanes 1-3 male DNA, 4-6 female DNA), run alongside a 1kb ladder (L). River Usk salmon.

Common bands can be seen present in all of the samples run using the *One102ADFG* primers (lanes a-f), showing a lack of gender-specific differences. This lack of gender-specificity can also be seen in the samples run using the *One18ASC* primers (lanes 1-6), although the bands in lanes 2 and 3 are somewhat more difficult to see.

There are common bands seen in all samples run using the *One102ADFG* primers, indicating that there are no gender-specific differences. Similarly, for the reactions using the *One18ASC* primers, although the bands seen were very faint they are present in all samples tested regardless of gender, therefore indicating that gender-specific differences are also lacking in the reactions using the O*ne18ASC* primers.

Fig. 4.1.2.iv shows an agarose gel of the products of the PCR reactions using the *Sal1UoG* primers, with DNA sampled from three male (lanes 1, 2, 3) and three female (lanes 4, 5, 6) individuals. The products were run alongside a 1kb ladder (L). The results show that although bands can be seen in individual tracks, there are no patterns evident with regards to exclusive presence in either gender, indicating that gender-specific differences do not exist for this sequence.

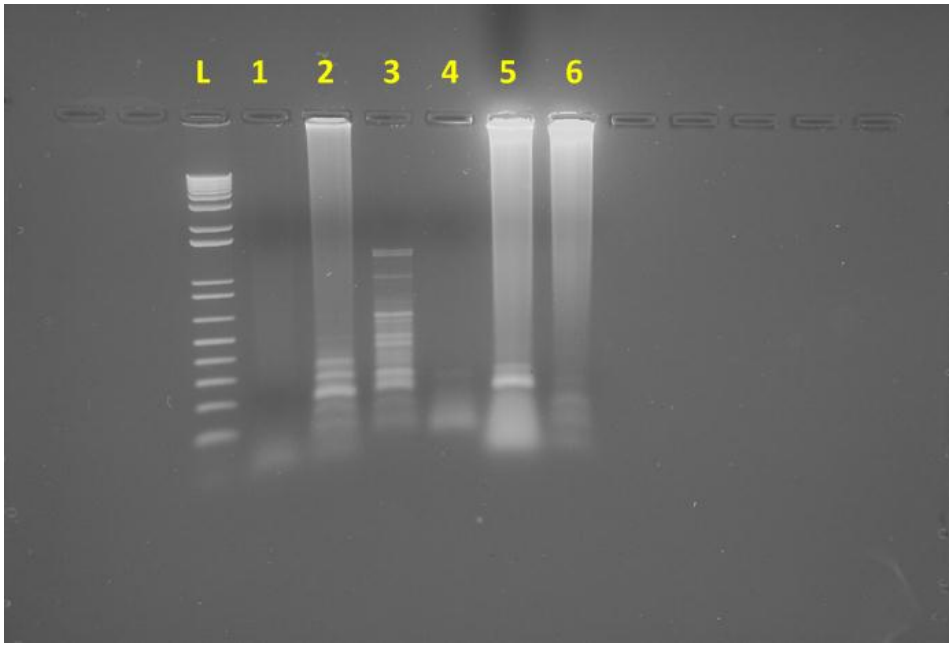


Fig. 4.1.2.iv

Agarose gel showing products of PCR reaction using *Sal1UoG* primers and male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). River Usk salmon.
Bands can be seen in the products of some of the samples (clearly in lanes 2,3,5; very faintly in lanes 4 and 6; none appsarent in lane 1), however these results represent differences between individuals, and not between gender in the presence of the *Sal1UoG* sequence.

Following on from tests on the River Usk race of Atlantic salmon, work commenced on testing in a second strain – the MOWI strain of salmon, with tissue samples provided by a commercial supplier. The same experimental method and PCR protocols were used, and DNA was extracted using the same methods as for the River Usk salmon.

Fig. 4.1.2.v shows the agarose gel of the PCR test using DNA from three male (lanes 1, 2, 3) and three female (lanes 4, 5, 6) MOWI salmon and the *Omy11INRA* primers, run alongside a 1kb DNA ladder.

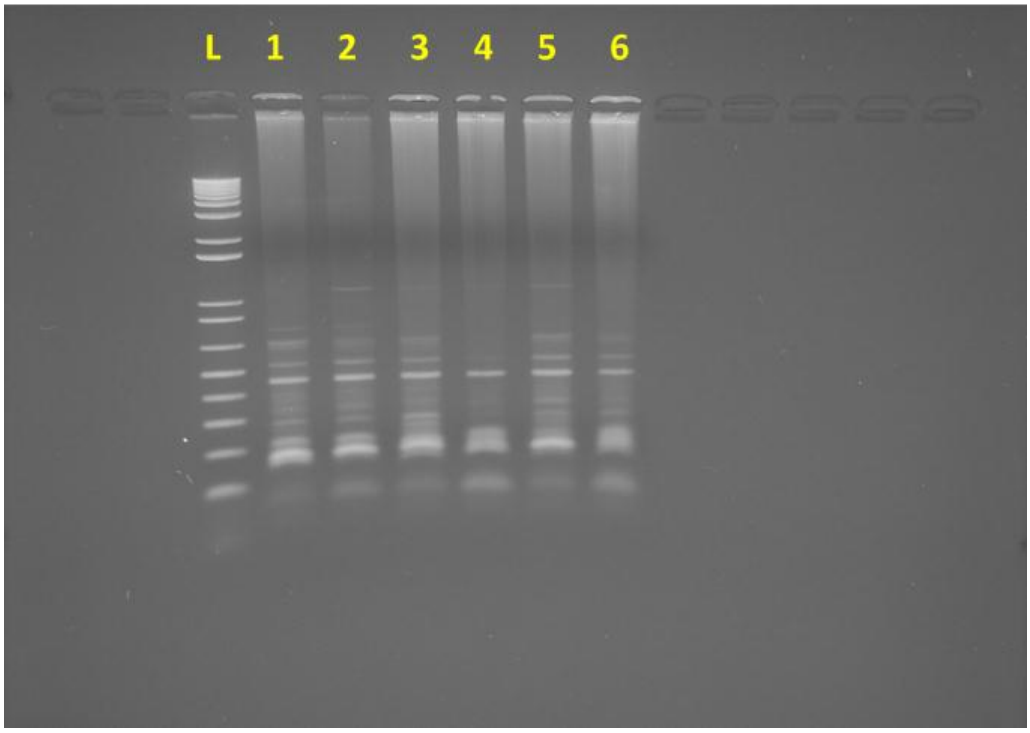


Fig. 4.1.2.v

Agarose gel showing products of PCR reaction using *Omy11INRA* primers and male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon. Bands can be seen that are common to all samples, whether they male DNA was used, or female. Although there are bands that are perhaps unique to individuals, this shows that the *Omy11INRA* sequence is present in both male and female DNA.

The results show that unlike the River Usk salmon, *Omy11INRA* is present in both male and female individuals of MOWI salmon, indicating that despite being able to use this sequence as a female-specific marker in the River Usk race, this would not be appropriate for the MOWI strain.

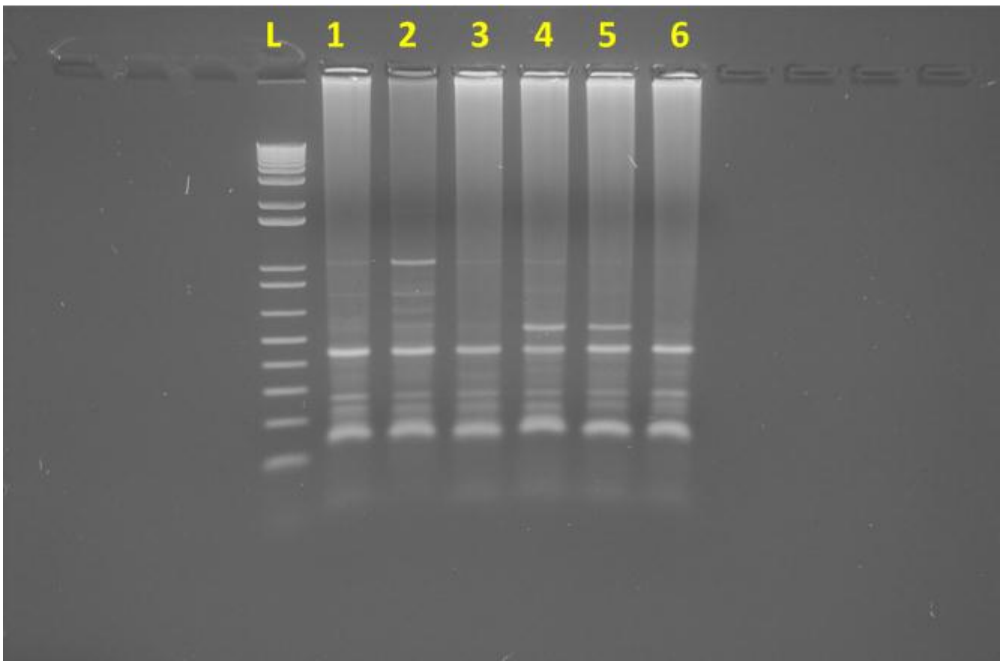


Fig. 4.1.2.vi

Agarose gel showing products of PCR reaction using *OmyFGT8TUF* primers and male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon. Strong common bands can be seen at ~420bp in the products for all samples of DNA tested, showing no gender specificity for the *OmyFGT8TUF* sequence. Additional bands can also be seen in all samples, but again there are no patterns relating to gender specificity.

The gel in Fig. 4.1.2.vi shows the PCR products for *OmyFGT8TUF* in three male (lanes 1-3) and three female (lanes 4-6) MOWI salmon, run alongside a 1kb DNA ladder (L). The gel shows that once again, the sequence was amplified in all tested individuals, confirming that no gender-specific differences exist in this sequence in this strain of salmon. There are also to be bands that appear to be specific to individuals.

Fig. 4.1.2.vii shows an agarose gel of the products for *Sal1UoG* in the same complement of male (lanes 1-3) and female (lanes 4-6) MOWI salmon. The results

again indicate a lack of gender-specificity, as shown by the presence of bands in every sample tested, and the presence of individual-specific bands is again apparent.

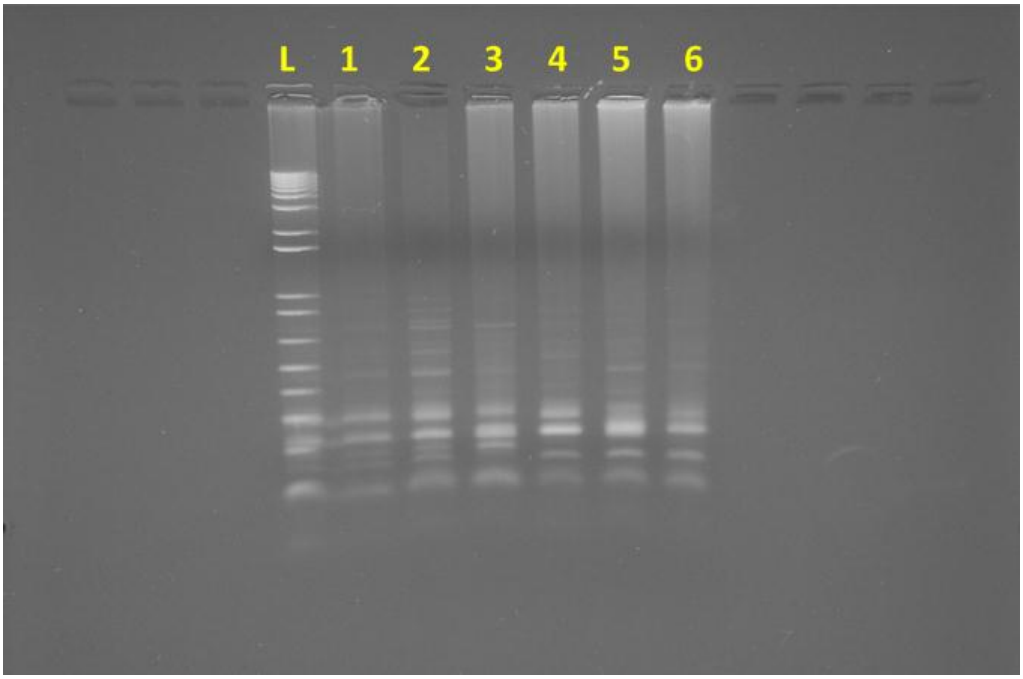


Fig. 4.1.2.vii

Agarose gel showing products of PCR reaction using *Sal1UoG* primers and male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon.
Common bands can be seen at ~200bp and ~300bp in the products for all samples of DNA tested, showing no gender specificity for the *Sal1UoG* sequence. Additional bands can also be seen in all samples, but again there are no patterns relating to gender specificity.

Fig. 4.1.2.viii is an agarose gel showing the products for the *One18ASC* primers in three male (lanes 1-3) and three female (lanes 4-6) MOWI salmon. The results indicate a lack of gender-specificity, as bands can be seen in corresponding positions for both male and female samples. Again, bands can be seen that are specific to individuals.

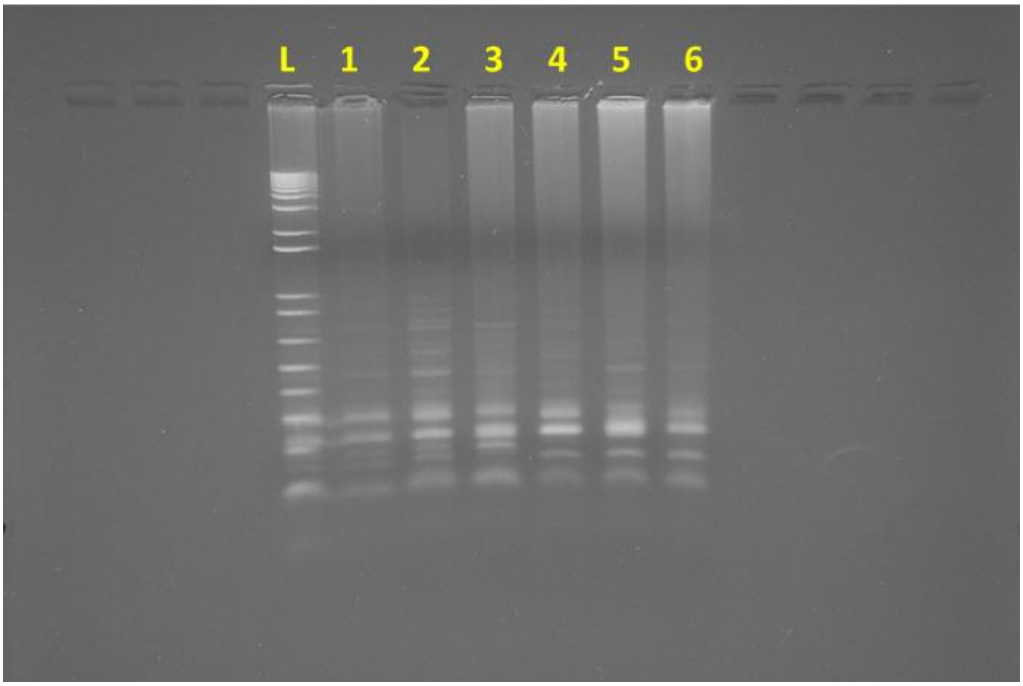


Fig. 4.1.2.viii

Agarose gel showing products of PCR reaction using *One18ASC* primers and male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon. Common bands can be seen at ~200bp and ~300bp in the products for all samples of DNA tested, showing no gender specificity for the *One18ASC* sequence. Additional bands can also be seen in all samples (most notably in lanes 2, 3, 5), but again there are no patterns relating to gender specificity.

The gel in Fig. 4.1.2.ix shows the PCR reaction products for the *One102ADFG* primers with three male (lanes 1-3) and three female (lanes 4-6) MOWI salmon, run alongside a 1kb ladder (L). Once again, the results show a lack of gender-specificity and the presence of bands specific to individuals.

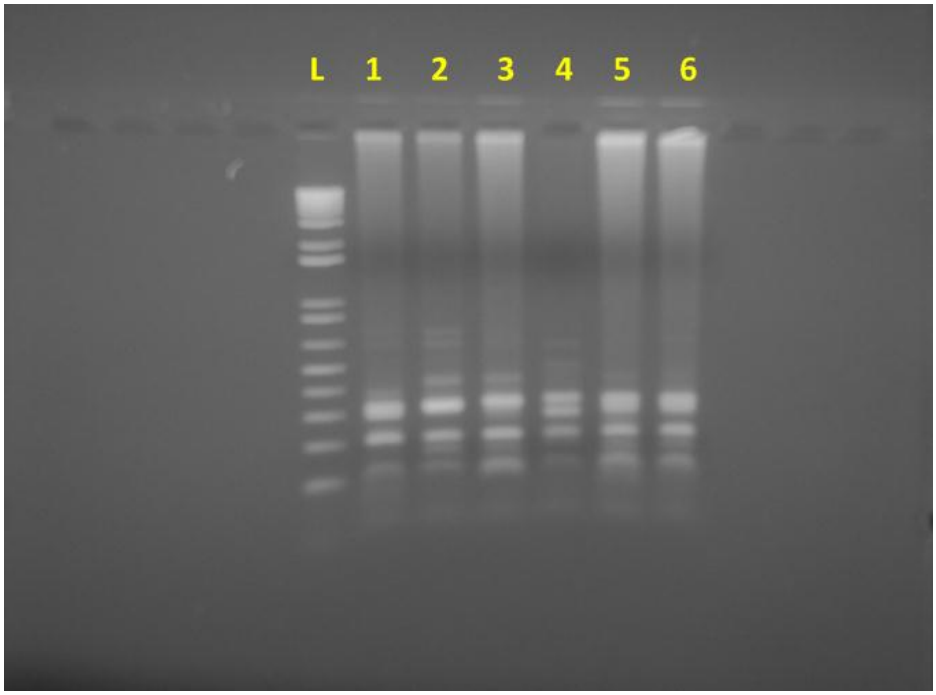


Fig. 4.1.2.ix

Agarose gel showing products of PCR reaction using *One102ADFG* primers with male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon. Common bands can be seen at ~200bp and ~300bp in the products for all samples of DNA tested, showing no gender specificity for the *One102ADFG* sequence. Additional bands can also be seen in all samples, but again there are no patterns relating to gender specificity.

Fig. 4.1.2.x and Fig. 4.1.2.xi are agarose gels showing the PCR products of the *Ssa406UoS* and *Str4INRA* primers respectively, each using DNA from three male (lanes 1-3) and three female (lanes 4-6) MOWI salmon, run alongside a 1kb ladder (L). The results of both show, consistently with all other PCR tests involving MOWI salmon in the present study, a lack of gender-specificity, and the presence of bands specific to individuals.

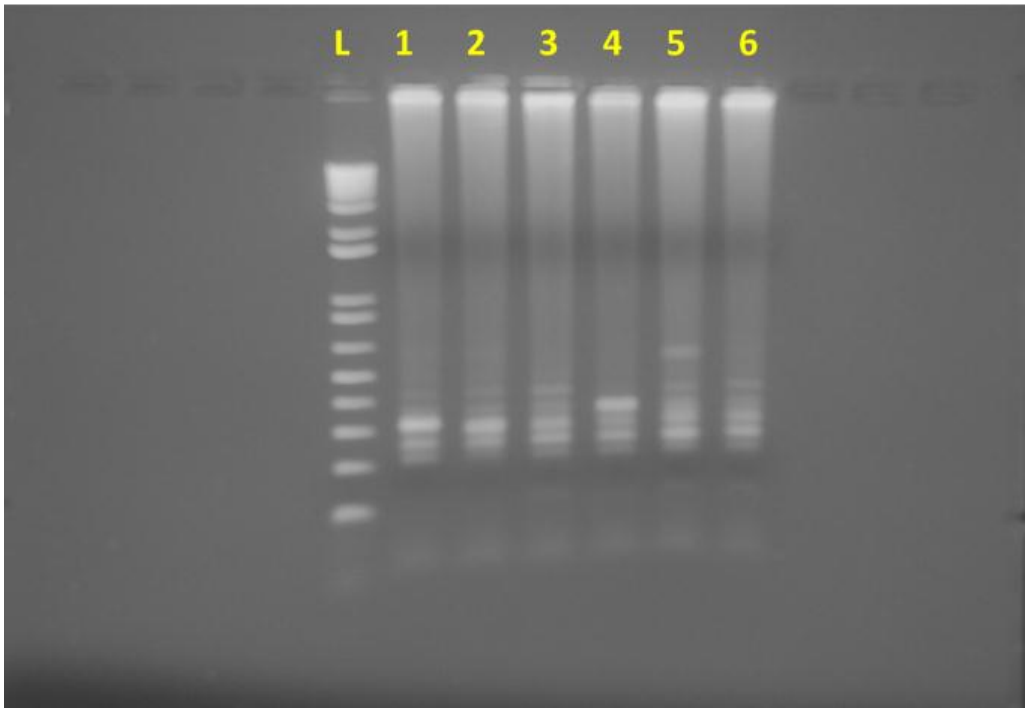


Fig. 4.1.2.x

Agarose gel showing products of PCR reaction using *Ssa406UoS* primers with male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon.

A number of bands can be seen for each sample, ranging from ~200bp to ~550bp, but there are no bands, or patterns of bands consistent in the samples for one gender but not the other.

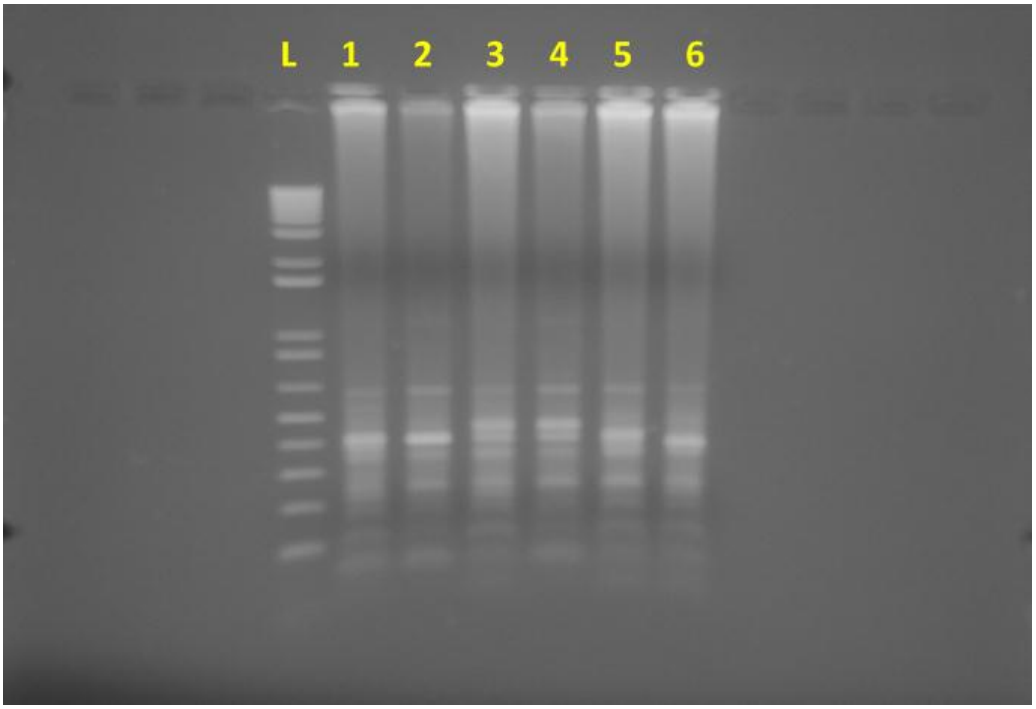


Fig. 4.1.2.xi

Agarose gel showing products of PCR reaction using *Str4INRA* primers with male (lanes 1-3) and female (lanes 4-6) Atlantic salmon DNA, run alongside a 1kb ladder (L). MOWI salmon. Multiple bands can be seen for each sample, with common bands to all in evidence at ~590bp and ~250bp. No clear evidence can be seen that might indicate gender-specific differences in the presence of the *Str4INRA* sequence in male and female MOWI Atlantic salmon DNA.

On analysis of the PCR products of the seven sequences, using MOWI salmon DNA, it would appear that none of the sequences tested have any differences between males and females of the MOWI strain of Atlantic salmon.

The third strain of salmon tested came from the Burrishole River in Ireland. DNA extraction and purification, and PCR protocols were the same as for the work done on the River Usk, and MOWI strains. However, resources were limited to a total of five samples of each gender in this strain, so all ten samples were tested simultaneously for

each of the sequences of interest. The agarose gels of the products of the PCR reactions can be seen below.

Key:

(L) 1kb DNA ladder

(1) Male 1

(2) Male 2

(3) Male 3

(4) Male 4

(5) Male 5

(6) Female 1

(7) Female 2

(8) Female 3

(9) Female 4

(10) Female 5

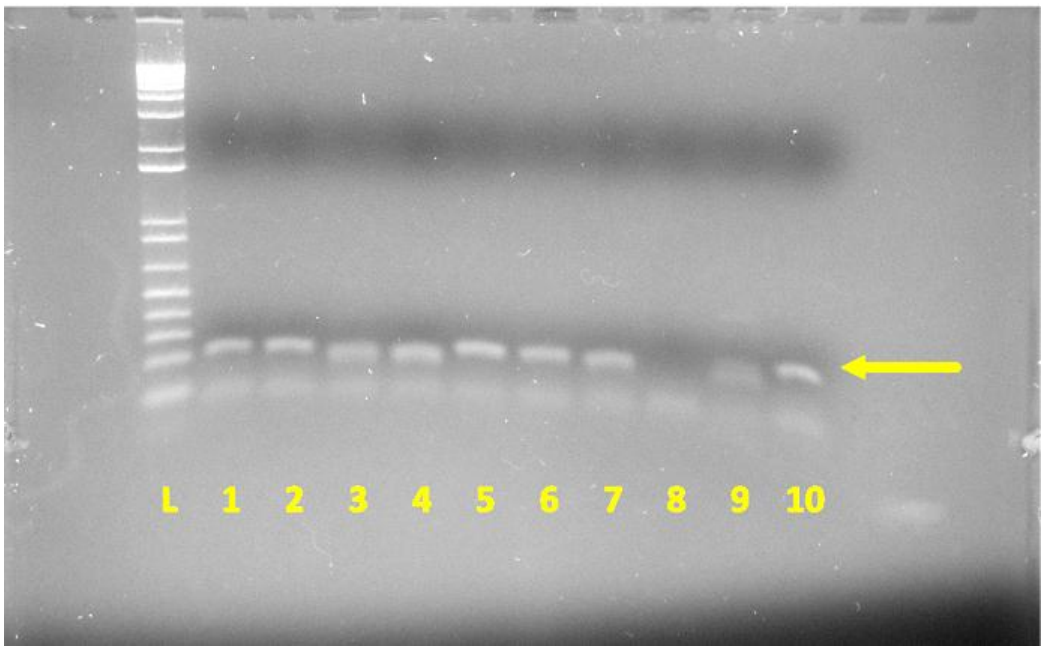


Fig. 4.1.2.xii

Agarose gel showing products of PCR reaction using *OmyFGT8TUF* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon, run alongside a 1kb ladder (L). Burrishole race.

Products can be seen in all samples at ~200bp apart from lane 8, indicating that the *OmyFGT8TUF* sequence shows no gender-specificity in the Burrishole race of Atlantic salmon.

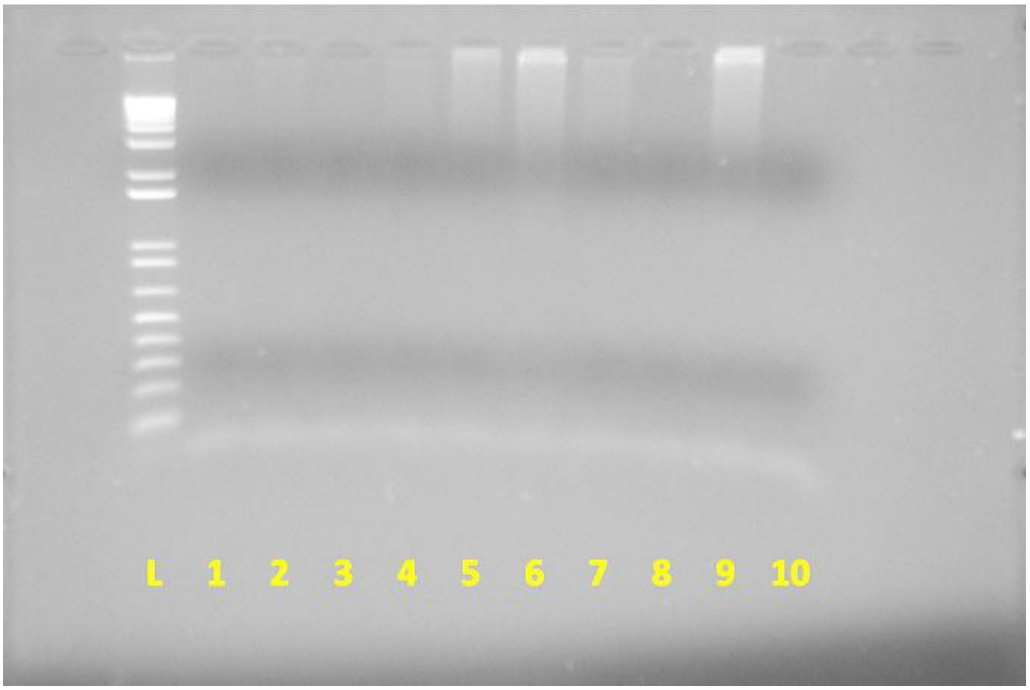


Fig. 4.1.2.xiii

Agarose gel showing products of PCR reaction using *One102ADFG* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon DNA, run alongside a 1kb ladder (L). Burrishole race.

There is no evidence of successful amplification in any of the samples, which may indicate that this sequence is not present in this race of Atlantic salmon.

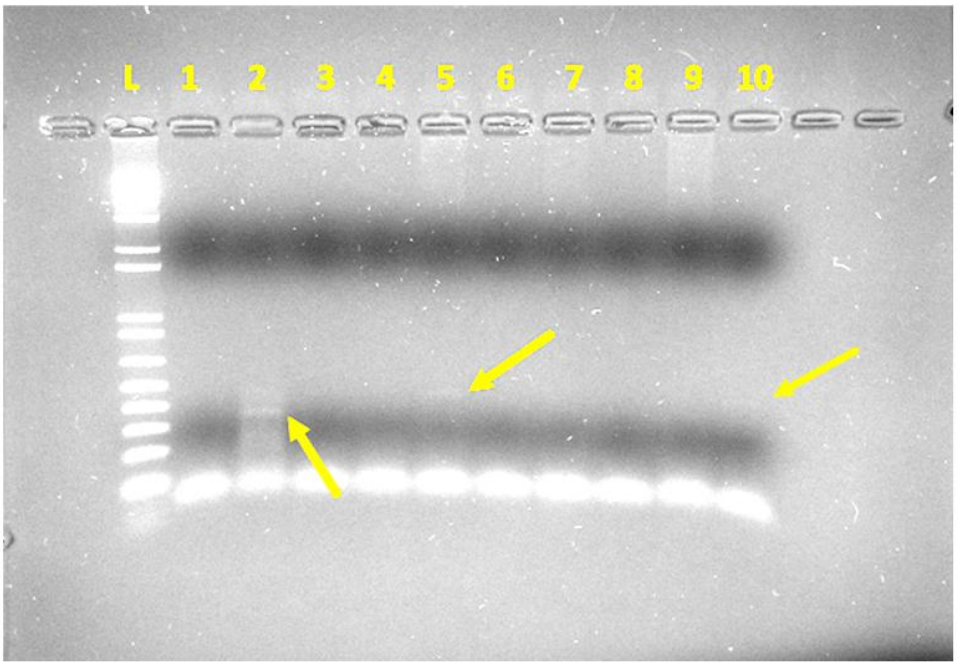


Fig. 4.1.2.xiv

Agarose gel showing products of PCR reaction using *Str4INRA* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon DNA, run alongside a 1kb ladder (L). Burrishole race. Bands can be seen in only three samples – at ~350bp in lanes 2 (male) and 10 (female), and ~450bp in lane 5 (male). The presence of bands in both male and female samples indicate that the *Str4INRA* sequence does not segregate with gender in this race of Atlantic salmon.

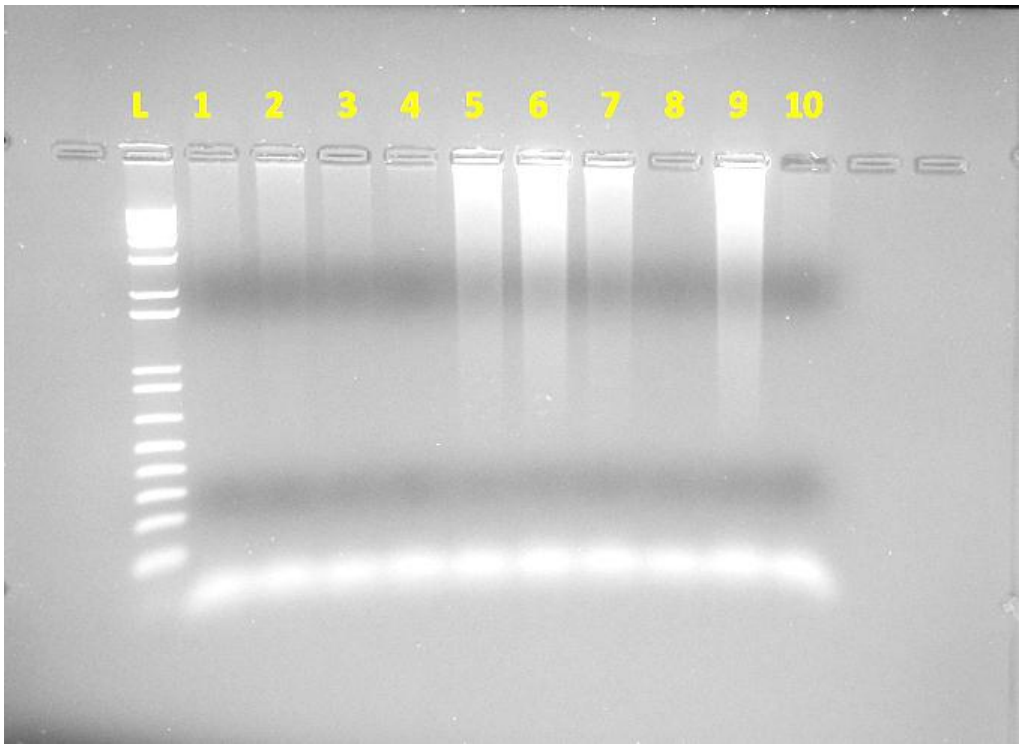


Fig. 4.1.2.xv

Agarose gel showing products of PCR reaction using *One18ASC* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon DNA, run alongside a 1kb ladder (L). Burrishole race. There is no evidence of successful amplification in any of the samples, which may indicate that this sequence is not present in this race of Atlantic salmon.

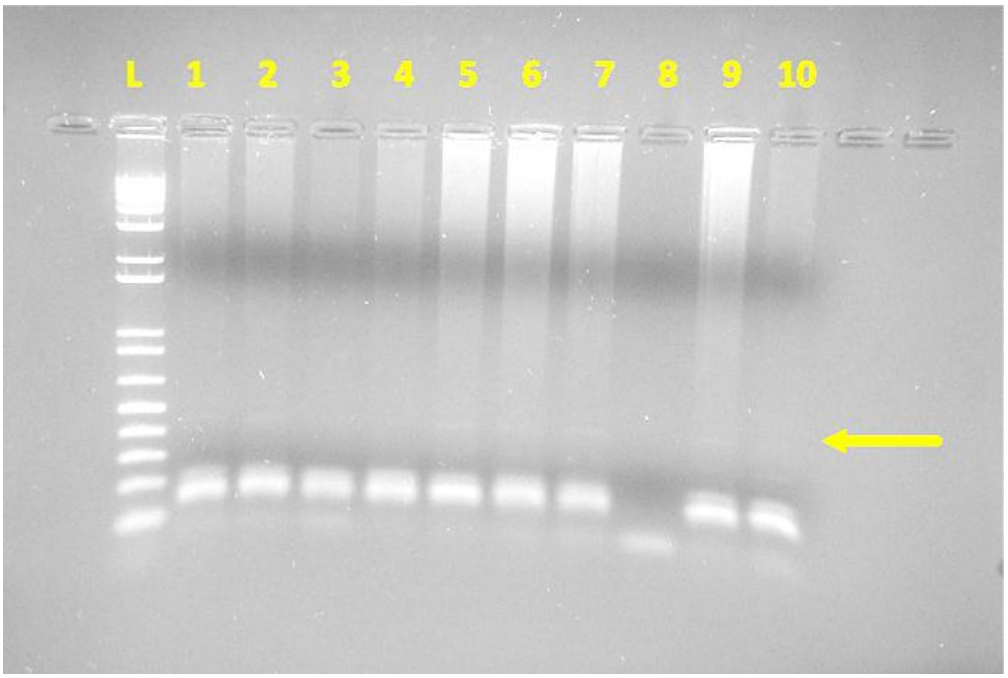


Fig. 4.1.2.xvi

Agarose gel showing products of PCR reaction using *Omy11INRA* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon, run alongside a 1kb ladder (L). Burrishole race.

Very faint bands can be seen at ~450bp for all samples apart from Female 3 and Female 5 (lanes 8 and 10), but this is not suggestive of any form of gender specificity.

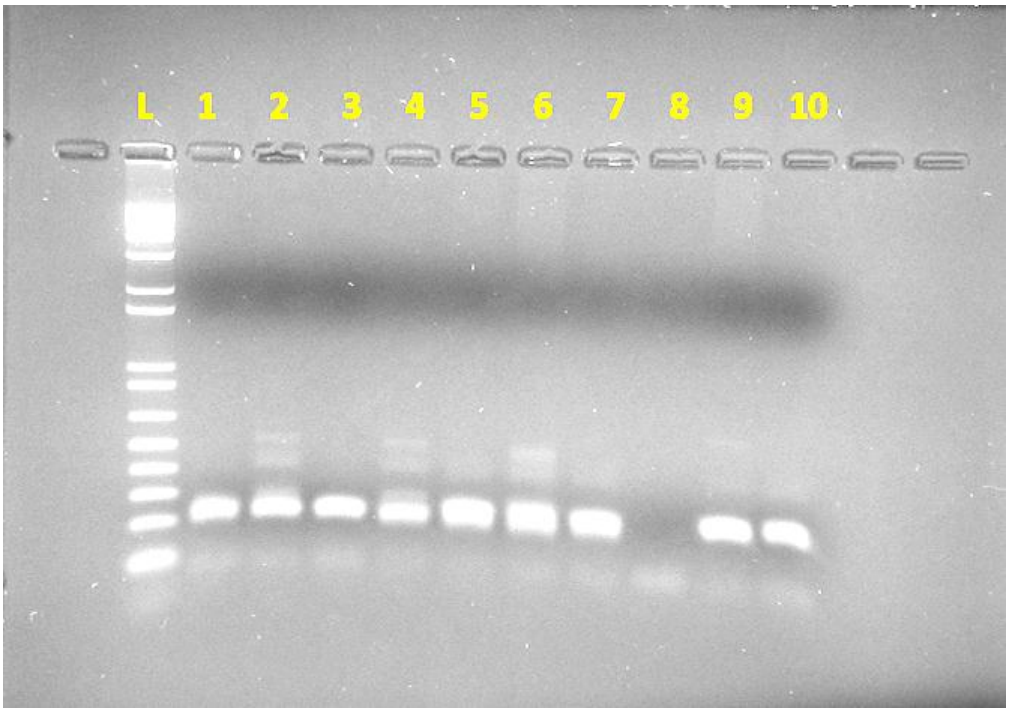


Fig. 4.1.2.xvii

Agarose gel showing products of PCR reaction using *Sal1UoG* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon DNA, run alongside a 1kb ladder (L). Burrishole race. Bands can be seen in samples 2, 4, 5, 6, 7 and 9, which correspond to males 2, 4 and 5, and females 1, 2 and 4. However, none of these appear to be specific to gender as for each band, a corresponding band can be found in at least one sample from the opposite sex. Thus, the *Sal1UoG* sequence does not segregate with gender in the Burrishole race of Atlantic salmon.

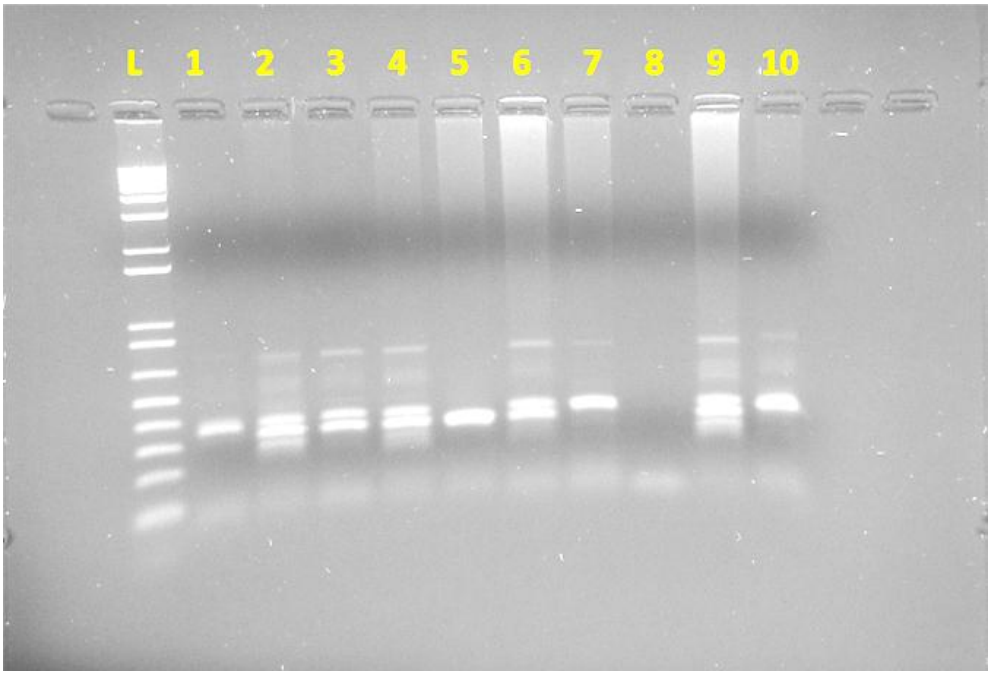


Fig. 4.1.2.xviii

Agarose gel showing products of PCR reaction using *Ssa406UoS* primers with male (lanes 1-5) and female (lanes 6-10) Atlantic salmon DNA, run alongside a 1kb ladder (L). Burrishole race. A clear band can be seen at ~630bp in three of the male samples (lanes 2, 3, 4) and four of the female samples (lanes 6, 7, 9, 10). Additional bands can also be seen in several of the samples, but there is no evidence to suggest that the *Ssa406UoS* sequence is gender-specific in the Burrishole race of Atlantic salmon.

The results show that no clear differences can be seen between genders for any of the sequences tested. In fact, in the gels for *One102ADFG* (Fig. 4.1.2.xiii) and *One18ASC* (Fig. 4.1.2.xv), it is unclear whether any reaction took place at all. There are amplifications of very similar size (~300bp) in nine out of the ten samples in Fig. 4.1.2.xii for *OmyFGT8TUF*, with Female 3 (lane 8) lacking in the band. A curious result can be seen in Fig. 4.1.2.xiv for *Str4INRA*, as there doesn't appear to be any amplification in any of the samples aside from the reaction with DNA from Male 2 (lane 2), and perhaps Male 5 (lane 5). In Fig. 4.1.2.xvi (*Omy11INRA*), there are very

faint bands at ~450bp for all samples apart from Female 3 and Female 5 (lanes 8 and 10). Fig. 4.1.2.xvii shows that amplification occurred in Males 2, 4 and 5 (lanes 2, 4, 5) and in Females 1, 2 and 4 (lanes 6, 7, 9), but none of these amplifications appear to be gender-specific, as each of the fragments have a corresponding result in at least one sample of the opposite sex. Fig. 4.1.2.xviii had the best resolution, and once again, multiple bands can be seen, though none indicate any gender-specificity. Similar to the gels for *Sal1UoG*, *Omy11INRA*, and *OmyFGT8TUF*, no evidence of amplification can be seen for Female 3 (lane 8) despite bands being present in all other samples on the gel.

4.1.3 Discussion

Taken together, the results of the PCR tests using the selected sequences identified one sequence – *Omy11INRA* – that can be used to differentiate between males and females, but only in the River Usk strain of Atlantic salmon, and as a positive marker for females, not males, as was expected. The corresponding tests using DNA from the MOWI and Burrishole strains of the species gave negative results – that is, no differences were identified between the male and female genders. Despite the lack of conservation across the different strains, this result gives one hope that there may yet be genes or sequences that are gender-specific to be found. The fact that this sequence is only gender-specific in one strain of Atlantic salmon means that there must be other similar and different mechanisms of sex determination in place within the species. Furthermore, the fact that it is not conserved in the three strains tested in this study

means that despite being sex-linked, *Omy11INRA* is certainly not a sex-determining factor.

Despite the early potential of gender specific markers in salmonids, particularly those of *OtY1* (Devlin et al., 1998) and *GH-ΨY* (Du et al., 1993) in various species of *Oncorhynchus*, none have since lived up to the promise of being the putative gender markers that many have searched for in this fascinating family of fish. Subsequent studies into these early candidates have shown that not only are they not conserved between species of salmonids, but they are also not even conserved within species. The early indications that *OtY1* was Y-chromosome specific in Chinook salmon (*Oncorhynchus tshawytscha*) were initially supported by later work (Stein et al., 2001; Devlin et al., 2001), but a study done on several different strains of Chinook salmon (Chowen & Nagler, 2005) has sinced showed that the reported specificity of *OtY1* to the Y-chromosome is lacking in strains other than the Columbia River population of Chinook salmon, on which the initial studies were based. *GH-ΨY* was also initially shown to be Y-specific in Chinook salmon, but was shown not to be conserved among all *Oncorhynchus* species (Devlin et al., 2001). Again, later work showed that *GH-ΨY* specificity is also not conserved in other strains of Chinook salmon outside of the Columbia River population (Chowen & Nagler, 2005). Y-chromosome variation has been reported between populations of rainbow trout (Phillips, 2001), leading to suggestions that this variation could potentially lead to speciation in the future. This is perhaps not surprising given that populations of migratory salmon (of both *Oncorhynchus* and *Salmo* species) generally return to their waters of birth in order to spawn, generation after generation, thus not mixing with salmon run from other rivers and streams, and consequently limiting the amount of gene flow between populations

of the same species. The results presented in the present study would, therefore, appear to be consistent with findings from other studies done in salmonids, in that while sequences may be found to be 'sex-linked' in certain strains of a particular species, they may well not be in other strains, and appear unlikely, even, to be conserved in other related species. One observation worth noting, however, is that in previous cases of apparent sex-specificity in salmonids, it is the male that the sequences are linked to. Interestingly in the present study, the only sequence found to be sex-specific (albeit only in one strain) is actually specific to females.

It is important to note that, while it is interesting from a scientific perspective to explore the ongoing evolution of sex chromosomes in salmonids, and by association, the mechanisms therein, the aims of this study do not require the elucidation of such mechanisms, nor that of the sex chromosomes in Atlantic salmon or salmonids in general. Specifically, the interest lies in whether differences exist on a genetic level in Atlantic salmon, and whether these differences can be used to develop a test that can be used in the field to positively identify males from females in this species. Based on these parameters, it is possible to conclude that there is a difference in the expression of the *Omy11INRA* sequence between the genders, namely that it is expressed in all of the females, and none of the males tested in this study. However, these differences lie only in the River Usk strain of Atlantic salmon, and therefore cannot be used to identify gender outside of it.

4.2 A different approach: Using Suppressive Subtractive Hybridisation to isolate gender-specific ESTs

The work described in section 4.1 has identified one sequence that shows differences in expression between genders (albeit only for one strain) in Atlantic salmon. However, with genomes being so large, it would perhaps be prudent to investigate differential sequences between genders across the entire Atlantic salmon genome. This forms the basis of the work presented in this sub-chapter. Presented here is a rationale behind the approach used in this strand of work, the findings from the work are reported and discussed. A comprehensive account of the materials and methods used can be found in the materials and methods chapter.

The main aim of the work conducted in this strand was to screen for differential sequences between gender in the genome of the Atlantic salmon. Clearly, attempting to do this using traditional PCR-based methods such as those employed previously in the current study would be immensely time-consuming and costly. Therefore, a newer, more innovative way of comparing two populations of genetic material and revealing differentially expressed sequences needed to be employed. Nisbet and Gasser (2004) used a suppressive subtractive hybridisation (SSH) approach to identify sex-specific genes in the nematode, *Trichostrongylus vitrinus*. They synthesised cDNA from RNA extracted from male and female individuals, then carried out expression profiling of gene libraries by microarray analysis. This enabled them to highlight gender differences and eventually allowed them to identify gender-specific genes. The aim of this strand of work is to use the suppressive subtractive hybridisation technique to

identify sequences that are in male Atlantic salmon DNA but not in female Atlantic salmon DNA, and sequences that are in the female DNA but not in the male.

The PolyA$^+$ RNA used in this study was extracted from total RNA using the TRIzol RNA Purification system (*Invitrogen*), and was stored at -80°C until required for use. The protocol used for the suppressive subtractive hybridisation (SSH) process was as specified by the PCR-Select cDNA Subtraction kit (*Clontech Laboratories Inc.*).

4.2.2 Results

The products from the suppressive subtractive hybridisation (SSH) procedure were run alongside a 1kb ladder on a 2% agarose gel containing ethidium bromide (5mg/ml) at 100V for 1 hour. The findings from the SSH work are shown below.

In Fig. 4.2.1, lanes 1 and 2 contain the products from the primary PCR stage (male in lane 1, female in lane 2), and lane 3 the product from the control primary PCR reaction. Lane 4 contains the products from the male secondary PCR reaction, and lane 5 the female secondary PCR reaction. Lane 6 contains the control secondary PCR reaction.

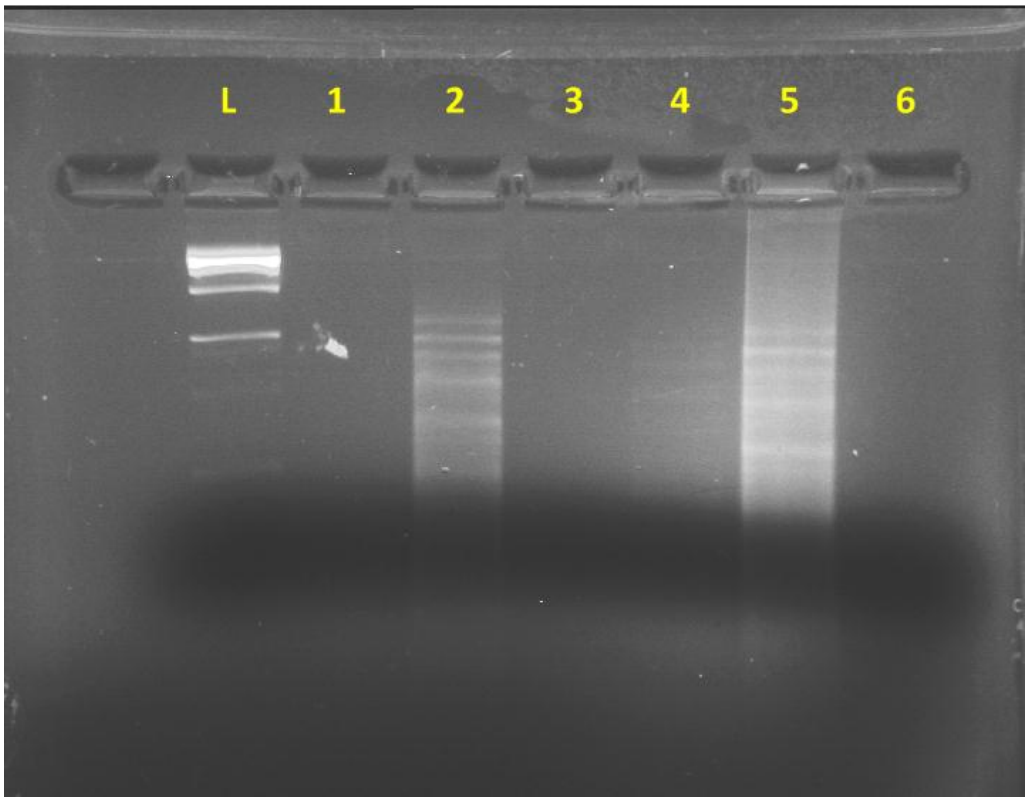


Fig. 4.2.1

Agarose Gel showing the primary and secondary PCR products from the SSH procedure. The primary PCR products are in lanes 1 (male) and 2 (female); lane 3 shows the products of the control primary PCR, and the secondary PCR products are in lanes 3 (male) and 4 (female). Bands can be seen in both of the secondary PCR products, indicating that differential sequences have been isolated from both male and female Atlantic salmon cDNA.

Very faint bands can be seen in lane 4, representing putative male-specific sequences, and clearer bands can be seen in lane 5, representing the putative female-specific sequences from the River Usk strain of Atlantic salmon. Unfortunately, it was difficult to examine these sequences as they were so faint, and so the process was repeated using more male cDNA. The products of the resulting secondary PCR reactions can be seen below (Fig. 4.2.2).

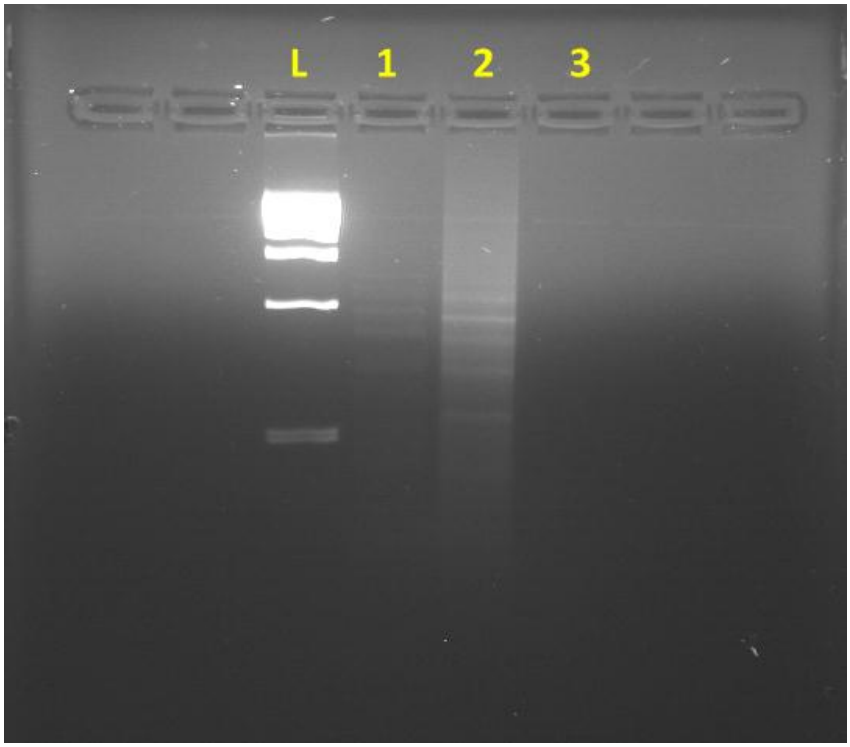


Fig. 4.2.2

Agarose Gel showing the secondary PCR products from the SSH procedure, with the male products in lane 1, female in lane 2, and control reaction in lane 3. Once again, the female sequences are visibly clearer.

Again, the male sequences (lane 1) are much fainter compared to the female sequences (lane 2), however this time is possible to observe five putative male-specific sequences, and seven putative female-specific sequences in the gel. As expected, nothing can be seen in the control (lane 3). To summarise, the aim of producing two populations of differentially expressed sequences – one specific to males, and one specific to females – from the River Usk strain of Atlantic salmon was a success.

Attempts were made at carrying out the SSH process with the MOWI and Burrishole strains of Atlantic salmon in order to find differentially expressed sequences in these strains for comparison, however isolating enough PolyA$^+$ RNA from total RNA proved problematic. Therefore, unfortunately this objective was not completed.

4.2.3 Discussion

Although the molecular processes involved in SSH are relatively straightforward, the physical task of conducting the work is extremely involved, and required a great deal of coordinating and timing. So much so in fact, that various stages required two persons working together. The SSH process is critically dependent on a supply of high quality PolyA$^+$ RNA, and so much time was spent on extracting high quality total RNA in the first instance, then on extracting the PolyA$^+$ RNA from the total RNA.

The resulting gel shows that there are indeed sequences present in the male DNA, that aren't in the female DNA, and sequences present in the female DNA that aren't in the male DNA. We know this because the SSH process selectively amplifies those sequences that are different between the two populations of DNA, and suppresses the amplification of those sequences that are the same. Furthermore, it appears that there are more sequences specific to female DNA than there are specific male DNA, although this is by no means conclusive. There also appears to be more genetic material present in all of the lanes where the products from female DNA have been separated. This may suggest a possibility that there are more genes switched on in the female genome than in males, at least at this stage of development. In turn, one could

perhaps hypothesise that males are in fact the ‘default’ gender in Atlantic salmon, with females being the heterogametic sex. Of course, there is no definitive evidence of this, and much more work would need to be done in order to verify these observations, and to provide more concrete evidence in support of this.

Unfortunately, limitations on both time and resources meant that the work using SSH, despite showing promise, was terminated before further progress could be made. Given more time and resources, the differential sequences could be studied in more detail. This would include cloning and sequencing the differential sequences to find out what they are, and mirroring these studies in the MOWI and Burrishole strains of Atlantic salmon to allow comparison of results with those obtained from the River Usk salmon. Examination of the differential sequences in these strains to see if any correspond to those found in the River Usk salmon would also be appropriate. It would also be interesting to determine whether there are in fact, more genes switched on in juvenile Atlantic female salmon than in males at the same stage in development, which would add support to the notion of female heterogamety in this species.

5. General Discussion

The ultimate aim of the current study was to develop a genetic test capable of positively distinguishing between males and females in a population of Atlantic salmon, *Salmo salar* from a small amount of tissue, namely an adipose fin clip. The reason for using the adipose fin is that it can be removed without causing the fish any harm, nor will it affect its stability in the water and therefore its ability to swim. The work undertaken was done in three strands, each as a stand-alone study, but the findings in each strand also contribute to the overall findings of the project.

The first task was to generate from wild samples, DNA of molecular grade quality suitable for investigative work. This proved to be a time-consuming process involving much optimisation, and although the resulting DNA was by no means ideal, it was of sufficient quality for further work to commence.

The next phase was to conduct tests to i) determine whether the *Sox9a* gene is present in the genomic DNA that was extracted and ii) to determine whether expression is specifically linked to gender. The *Sox* family of genes were of particular interest as several members of this family have been implicated in vertebrate sex determination and/or testis development. Of these, *Sox9* and its orthologues have been shown to play a central role in vertebrate testis development. *Sox9a* was chosen as the candidate sequence because of its role in testis formation in various fish species including rainbow trout, a related species of Atlantic salmon. As it turned out, this sequence did not appear to be gender-specific, as it was positively amplified in PCR reactions in

both male and female genomic DNA. Of course, this does not suggest that all of the *Sox* genes are not gender-specific, just *Sox9a*. However, it would be a very cumbersome and time consuming (and very expensive) process to investigate all of the *Sox* genes in this fashion. Furthermore, as there are many additional genes and sequences across the animal kingdom linked to sex determination, it would be necessary to test each of these too. Given the size, and of course, impracticalities of such a vast project, it would be astute to look for a more manageable approach.

Work done on certain species of Pacific salmon (genus *Oncorhynchus*) identified a number of sequences that held promise in the search for sex-specific markers in some salmonids (Devlin et al., 1998, Du et al., 1993). A number of these sequences were shown to be present in the Atlantic salmon genome (Woram et al., 2003) and moreover, located close to the putative sex-determining locus of Atlantic salmon. It was not reported whether these sequences were specifically linked to sex, and given their apparent proximity to the sex-determining locus in Atlantic salmon; it was of interest to elucidate whether they did segregate with gender in the species. The results were negative for the majority of these sequences, but one of the tested sequences, *Omy11INRA*, did show gender specificity, however it was specific for female DNA, and not male DNA. Furthermore, when this sequence was tested for the same specificity in two other strains of Atlantic salmon (MOWI, and Burrishole), this specificity was not apparent. Additionally, some of the sequences tested did not even amplify in some of the PCR reactions, suggesting that they did not exist at all in the DNA tested. These results show that the *Omy11INRA* sequence can be used to positively identify female individuals in the River Usk population of Atlantic salmon, but it cannot be used in other strains – at least not in the MOWI or Burrishole River

strains that were tested in the present study. Subsequent work on the putative genetic markers in Pacific salmon were later shown not to be gender-specific in any other strains of the particular *Oncorhynchus* species other than the one involved in the original study (Chowen & Nagler, 2005).

A comparison of the genome of male Atlantic salmon with that of the female counterpart would be extremely useful in identifying differential sequences between the two, but this traditionally would have meant doing a gene by gene comparison of the two populations of DNA. Clearly, this would have been an almost impossible task, given that the smallest vertebrate genome consists of 342 000 000 bp (Green pufferfish, *Tetraodon fluviatilis*), and the largest vertebrate genome 129 907 000 000 bp (Marbled lungfish, *Protopterus aethiopicus*) (Gregory, 2005). Advances in technology and improvements in the techniques used have allowed work to be done that perhaps wasn't possible before. One of these, suppressive subtractive hybridisation allows the comparison of two populations of mRNA and can yield sequences expressed in one population but not the other. This technique was employed in the present study and resulted in the obtainment of two sets of differentially expressed sequences – one from male genetic material, and one from female genetic material. One observation that can be made from the resulting gels is that there appears to be more genetic material present in the lanes relating to female DNA. Whilst the evidence is entirely circumstantial, there is the possibility of this suggesting that females are the heterogametic sex, not males. In fact, this would explain in part the results observed in the work done with the *Omy11INRA* sequence as described above. If females were the heterogametic sex in Atlantic salmon, it would make sense that if any genuine genetic markers were to be found; they would be

female-specific and not male-specific. There are already many examples of female heterogamety in other species of fish (Mank et al., 2006). It is also possible that there are no differences at all on a genetic level, and that sex determination of Atlantic salmon is entirely based on environmental factors, as is the case in many reptiles and some fish, although a recent study suggests that this is far less widespread than is generally believed to be (Ospina-Álvarez, 2008)

Another point worthy of mention is that the genomic DNA used is essentially extracted from wild populations. There is a possibility (however small) that the positive PCR amplification seen using the *Omy11INRA* primers with DNA from the River Usk salmon represents amplification of DNA alien to the Atlantic salmon – that is, DNA from another organism (perhaps that of a parasite, or another organism living on the body of the animal in question).

In summary the work presented in this thesis suggests that it is possible to differentiate between male and female juvenile Atlantic salmon, at least for the River Usk race. This potentially opens up a range of exciting research possibilities. To the extent that the River Usk race is subject to the same ecological pressures as other Atlantic salmon, it may become possible to address questions about how these amazing fish take crucial life history decisions such as which gender to adopt, and how these are affected by pressures from environmental change. It has become increasingly fashionable to treat the natural world and its inhabitants as victims of the insatiable consumerism and even thoughtless actions of "unnatural" humankind, but this can be seen as being imprudent, perhaps even hubristic in respect of humankind, and mawkish in respect of the Natural World. Application of the tool developed in this

thesis, without preconceptions concerning humankind's deleterious meddling, will perhaps reveal the extraordinary subtlety with which the vigorous and successful Atlantic salmon is dealing with the latest novel selection pressure in its long evolutionary history.

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